MYCOBACTERIUM TUBERCULOSIS GENOTYPES AND THEIR RELATIONSHIPS WITH CLINICAL AND IMMUNOLOGICAL PHENOTYPES IN SINGAPORE

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TABLE OF CONTENTS

SUMMARY

This PhD thesis consists of several retrospective and prospective studies in molecular epidemiology as well as in genotype and phenotype relationships of *Mycobacterium tuberculosis*. The studies presented in chapters 2 and 3 aimed to uncover the genetic diversity and population structure of *M. tuberculosis* and to formulate a strain-typing strategy for *M. tuberculosis* in Singapore. We analyzed 364 consecutively collected drug-susceptible *M. tuberculosis* isolates using IS*6110* restriction fragment length polymorphism (RFLP) typing, spoligotyping, and mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing. We found that all the seven major worldwide prevalent families of *M. tuberculosis*, *i.e*. the Beijing family (53.8%), the East-African-Indian (EAI) family (21.7%), the Haarlem family (8%), the Latin-American-Mediterranean (LAM) family (1.6%), the Central Asia (CAS) family (0.5%), the T family (9.1%), and the X family (0.8%), were present in Singapore. Moreover, a novel evolutionary clone was identified and designated as "S" family (4.5%). These data showed the high genetic diversity of *M. tuberculosis* and the predominance of the Beijing genotype in Singapore. Among the three typing methods, no single method could differentiate all unique isolates. We then analyzed the discriminatory power of different combinations of the three methods. The combination of IS*6110* RFLP and MIRU-VNTR typing showed the highest discriminatory power. A two-step strain-typing strategy has therefore been proposed that uses MIRU-VNTR typing as first line screening method and IS*6110* RFLP typing as secondary typing modality for MIRU-VNTR defined clusters. This typing strategy would greatly reduce typing workload and provide 'real-time' results for most isolates.

The study presented in chapter 4 aimed to examine the relationship between *M. tuberculosis* Beijing genotype strains and tuberculosis relapse. Our results showed that the Beijing genotype was associated with tuberculosis relapse in Singapore (odds ratio, 2.64; $p = 0.005$).

The study presented in chapter 5 aimed to understand the transmission dynamics of drug-resistant tuberculosis and relationships between genotypes and drug-resistant phenotypes of *M. tuberculosis*. We analyzed a population sample of 234 drug-resistant isolates using genotyping methods. We found that the Beijing genotype (odds ratio, 2.61; $p = 0.017$) and resistance to streptomycin (odds ratio, 2.01; $p = 0.044$) were risk factors for clustering and that only about 11% of drug-resistant tuberculosis was due to recent transmission. In addition, we also found that there were several significant positive and negative associations between *M. tuberculosis* genotypes and drug-resistant phenotypes. These data suggest that the transmission of drug-resistant tuberculosis is low in Singapore and different genotypes of *M. tuberculosis* may have different preference in the development of drug-resistant patterns.

The study presented in chapter 6 aimed to investigate whether Beijing genotype strains elicit a weaker Th1 immunity and are clinically more virulent in human tuberculosis. By clinically and immunologically comparing tuberculosis associated with Beijing and non-Beijing strains, we found that patients in the Beijing group were characterized by significantly lower frequency of fever (odds ratio, 0.12; $p = 0.008$) and pulmonary cavitation (odds ratio, 0.2; $p = 0.049$). Night sweats were also significantly less frequent by univariate analysis, and the duration of cough prior to diagnosis was longer in Beijing compared to non-Beijing groups (medians, 60 versus 30 days, $p =$

0.048). The plasma and gene expression levels of IFN- γ and IL-18 were similar in the two groups. However, patients in the non-Beijing group had significantly increased IL-4 gene expression ($p = 0.018$) and lower IFN-γ : IL-4 cDNA copy number ratios ($p = 0.01$). These findings suggest that patients with tuberculosis caused by Beijing strains appear to be less symptomatic than those who have disease caused by other strains. Th1 immune responses are similar in patients infected with Beijing and non-Beijing strains but non-Beijing strains activate more Th2 immune responses compared with Beijing strains, as evidenced by increased IL-4 expression.

LIST OF TABLES

LIST OF FIGURES

Figure 6.14. IFN-γ : IL-4 cDNA copy number ratio of patients with cavitary versus non-cavitary tuberculosis.

ABBREVIATIONS

µl	microliter
µM	micromolar
µg	microgram
AIDS	acquired immunodificiency disease syndrom
bp	base pair
cDNA	complementary DNA
cm	centimeter
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide-5'-triphosphate
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	gram
HIV	human immunodeficiency virus
IFN	interferon
IL	interleukin
kDa	kilodalton
L	liter
MIRU-VNTR	mycobacterial interspersed repetitive unit-variable number tandem repeat
mg	milligram
ml	milliliter
mM	millimolar
ng	nanogram

nm	nanometer
OD	optical density
p	probability
PCR	polymerase chain reaction
pmol	picomole
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolution per minute
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
TGF	transforming growth factor
TNF	tumor necrosis factor

CHAPTER 1

LITERATURE REVIEW

1.1 History and Epidemiology

Tuberculosis is an ancient human disease caused by bacterial infection and remains among the top 10 causes of death in the world today (Bleed et al., 2000). Molecular evidence from the Egyptian and South American mummies date it back to thousands of years ago (Salo et al., 1994; Nerlich et al., 1997). The disease became epidemic first in Europe about 400 years ago. Europe became the epicenter of many tuberculosis epidemics from the 16th century onwards due to population expansion, industrialization, and development of large urban centers. In the 18th-19th centuries, tuberculosis was the major cause of death in Europe. It is estimated that one-quarter of Europeans died of the disease. In 1882, Robert Koch first identified the etiological agent of tuberculosis, *Mycobacterium tuberculosis*, from patient's sputum. It has been long thought that tuberculosis was spread into Europe followed the immigration of Indo-European cattle herders who were infected by tubercle bacillus, *M. bovis* or an adaptor, from cattle (Bates and Stead, 1993). However, this hypothesis has been refuted by a very recent study in which the new findings suggest that *M. bovis* is the final evolutionary member of a diverged lineage from *M. tuberculosis* which includes *M. africanum*, *M. microti*, and *M. bovis*. This novel evolutionary scenario for *M. tuberculosis* complex suggests that *M. tuberculosis* is the progenitor of *M. bovis* (Brosch et al., 2002).

Tuberculosis peaked in Europe in the first half of the 19th century. It declined in the latter half of the 19th century largely because of socioeconomic improvements and possibly also because of the isolation of infectious cases. The declining trend was maintained throughout most of the 20th century and accelerated by the widespread vaccination of an attenuated Bacille Calmette-Guerin (BCG) vaccine and the application

of antituberculosis agents in the latter half of the 20th century. This resulted in neglect to the epidemic and the wane of necessary public health infrastructure for tuberculosis control for a period of time in developed countries (Maher and Raviglione, 2005) despite it was still a problem in the developing world.

Tuberculosis was spread to the other regions of the world by European immigration and colonization in the 400 years from the 16th-19th centuries. It reached peak about a century later in Asia than was in Europe, even later in some other areas, such as, Papua New Guinea, Indonesia, and the sub-Sahara of Africa (Bates and Stead, 1993; Smith, 2003). The epidemic has since been a public health problem in developing countries.

The declining trend in developed countries, however, has been reversed since the mid-1980s and the disease has re-emerged as a major killer worldwide. In 1993, the World Health Organization (WHO) declared tuberculosis a global emergency. It is estimated by the WHO that one third of the global population is infected by *M. tuberculosis*, with approximately 8 million new tuberculosis cases and 2 million deaths reported annually (Corbett et al., 2003). Developing countries bear the brunt of the tuberculosis epidemic, about 95% of the world's tuberculosis cases and 98% of the tuberculosis deaths occur in the developing countries (Maher and Raviglione, 2005). The top 22 high-burden countries accounted for roughly 80% of the world's tuberculosis cases in 2002 (WHO, 2004; Maher and Raviglione, 2005). For example, India alone takes 20% of the burden. Poverty, HIV pandemic, malnutrition, poor health care, and lack of adequate tuberculosis control are among the factors that are responsible for this heavy burden in the developing world. HIV infection has emerged as the most important risk

factor for progression of dormant *M. tuberculosis* infection to active disease and for contracting the disease from new infection in the sub-Sahara Africa and some other areas of the world (Maher and Raviglione, 2005). In developed countries, tuberculosis is a disease occurring mostly in some specific groups of persons, such as the homeless, foreign-born immigrants from countries with high tuberculosis incidence, and also HIV/AIDS patients.

To effectively control the epidemic, the WHO has promoted a global five-element strategy, called directly observed treatment by standard short-course anti-tuberculosis therapy (DOTS) in 1993 (Maher and Mikulencak, 1999; WHO, 2002). The five elements of DOTS include:

1. Sustained government commitment to tuberculosis control;
2. Diagnosis based on quality-assured sputum-smear microscopy mainly among symptomatic patients presenting to health services;
3. Standardized short-course chemotherapy for all cases of tuberculosis, under proper case-management conditions including direct observation of treatment;
4. Uninterrupted supply of quality-assured drugs;
5. A standard recording and reporting system enabling program monitoring by systematic assessment of treatment outcomes of all patients registered.

Despite the global efforts, the two targets of the DOTS program (to detect at least 70% of all smear-positive tuberculosis cases, and to treat successfully at least 85% of the detected smear-positive tuberculosis case) set for the period of 1993 to 2000 was not reached and has been re-set to 2005 (WHO, 2000a). The achievement of the two target percentages of the DOTS would eventually reduce both the prevalence of infectious

tuberculosis cases and the number of infected contacts by about 40% and would lead to an expected decline in annual tuberculosis incidence rate of 6% to 7% per year, and by 2015 to have halted and begun to reverse the incidence and death rates of tuberculosis (Maher and Raviglione, 2005).

1.2 The *M. tuberculosis* Complex

The *M. tuberculosis* complex (MTC) consists of a group of acid-fast mycobacteria which cause tuberculosis diseases in a wide range of mammalian hosts (Cole, 2002). It comprises five classical species. The species *M. tuberculosis* and *M. africanum* are human pathogens, but infections by the two species have also been found in other primates and animals (Cole, 2002); unlike *M. tuberculosis*, *M. africanum* is prevalent only in equatorial Africa (Aranaz et al., 1999), although it has recently been isolated from African and Vietnamese immigrants in Europe and the United States (Viana-Niero et al., 2001; Desmond et al., 2004); *M. bovis* is the causative agent of bovine-type tuberculosis, infects a wide range of animal species and man (Morris et al., 1994; O'Reilly and Daborn, 1995); *M. bovis* BCG is a laboratory-attenuated vaccine strain of *M. bovis*, has been used extensively as a vaccine against human tuberculosis; *M. microti* is the cause of vole tuberculosis, almost exclusively a rodent pathogen and has been successfully used as a live vaccine (Frota et al., 2004). Recently, three new members of the MTC, *M. canettii* (van Soolingen et al., 1997), *M. pinnipedii* (Cousins et al., 2003), and *M. capare* (Aranaz et al., 1999, 2003) have been reported, and were found to affect a variety of mammals, but mainly cause diseases in humans, seals, and goats respectively. The MTC members therefore differ greatly in their host tropisms, phenotypes, epidemiology, pathogenesis,

and in some biochemical characteristics; for example, *M. bovis* isolates are naturally resistant to pyrazinamid, whereas *M. capare* isolates are sensitive to the drug (Aranaz et al., 1999).

Genetically, however, there has been extensive experimental evidence showing that the MTC bacilli are highly conserved. At individual gene level, the nucleotide sequences of 16S rRNA gene (Böddinghaus et al., 1990; Rogall et al., 1990, van Soolingen et al., 1997), the *dnaJ* gene (Takewaki et al., 1993), the 65 kDa heat-shock protein gene (Telenti et al., 1993), the internal transcribed spacer (ITS) region between 16S rRNA and 23S rRNA (Frothingham et al., 1994; Glennon et al., 1994), and many more other genes (Sreevatsan et al., 1997) are identical among the members of the MTC. At whole genome level, the species of the MTC share greater than 99% of DNA identity (Brosch et al., 2000). Furthermore, DNA sequence analysis of the MTC isolates have revealed that allelic polymorphism is extremely restricted, occurring in ~1 in 10,000 base pairs, significantly less compared to other pathogenic bacteria (Sreevatsan et al., 1997). As such, it has been suggested that the species of the MTC should be re-classified as subspecies of *M. tuberculosis* (van Soolingen, 2001; Mostowy et al., 2002).

1.3 DNA Fingerprinting Methods of *M. tuberculosis*

There have been a great number of methods for *M. tuberculosis* genotyping developed in the last fifteen years (Kremer et al., 1999; van Soolingen, 2001; Mazars et al., 2001). Among them, IS*6110* restriction fragment length polymorphism (RFLP) typing (van Embeden 1993), spoligotyping (Kamerbeek et al., 1997), and mycobacterial interspersed

repetitive unit-variable number tandem repeat (MIRU-VNTR) typing (Mazars et al., 2001) are the most widely used.

1.3.1 IS*6110* RFLP Typing

The mobile insertion sequence IS*6110* was first identified from the chromosome of *M. tuberculosis* in 1990 (Thierry et al., 1990a; McAdam et al., 1990) and was found to be specific to the members of the MTC (Hermans et al., 1990; Thierry et al., 1990b; Cave et al., 1991). It has since been widely used as a PCR diagnostic marker for tuberculosis (Hermans et al., 1990; Thierry et al., 1990b; Brisson-Noel et al., 1991) and a DNA fingerprinting marker of MTC isolates (Hermans et al., 1990; Thierry et al., 1990b; Cave et al., 1991).

IS*6110* RFLP typing is based on the difference of copy numbers, ranging from 0 to about 25, and variability in chromosomal positions of IS*6110* inserts between strains (Hermans et al., 1990; Cave et al., 1991). Three underlying mutational mechanisms, including IS*6110* insertion, chromosomal mutation, and deletion, may drive the IS*6110* RFLP diversity (Warren et al., 2000). IS*6110* RFLP typing is reproducible and highly discriminatory on population level (Kremer et al., 1999), and currently serves as a "gold standard" strain-typing technique for *M. tuberculosis* (van Soolingen 2001). However, IS*6110* RFLP typing has several disadvantages. It is a slow, cumbersome, labour intensive and technically demanding technique requiring relatively large amounts (≥ 2 μg) of high quality bacterial DNA, an amount that can only be extracted from a large number of bacteria obtained from subcultures of *M. tuberculosis*. The time to grow the bacteria usually takes weeks. Also, this method has very poor discriminatory power for

isolates with fewer than 6 of IS*6110* copies, and is not informative for isolates, though very rare, which do not have IS*6110* insert (Das et al., 1995; van Soolingen 2001). Finally, to facilitate interlaboratory comparison of IS*6110* RFLP patterns, a internationally standardized methodology has been recommended (van Embeden et al., 1993); despite this, it remains difficult to compare *IS6110* RFLP results between laboratories as sophisticated computer software are required to analyze IS*6110* RFLP patterns.

Therefore, in order to increase the discriminatory power of IS*6110* RFLP typing, a secondary strain-typing method is needed for the isolates with fewer than 6 IS*6110* copies. The most used methods for this purpose are spoligotyping (Bauer et al., 1999; Yang et al., 2001; Kwara et al., 2003), MIRU-VNTR typing (Cowan et al., 2002; Kwara et al., 2003), and the polymorphic GC-rich sequence (PGRS) RFLP typing (Yang et al., 2001).

1.3.2 Spoligotyping

Spoligotyping is based on the hybridization detection of the presence or absence of 43 distinct direct variant repeats (DVRs) in the direct repeat (DR) region of the bacillary genome, each DVR consists of an identical 36-bp direct repeat sequence and a variable (both in length, 35 to 41 bp, and in nucleotide sequences) spacer DNA sequence (Kamerbeek et al., 1997).

Spoligotyping is a PCR-based strain-typing method, thus need only a few bacteria that can be obtained from either the primary culture of the bacilli or directly from clinical specimens (Heyderman et al., 1998), or even from slides of Ziehl-Neelsen staining (van

der Zanden et al., 1998), making it a "real-time" analysis tool. Furthermore, this method is highly reproducible and easy to perform (Kremer et al., 1999); particularly spoligotypes can be digitized into binary or octal code formats (Dale et al., 2001) which greatly facilitate the management and interlaboratory comparison of spoligotyping data. An international spoligotyping database (SpolDB) has been set up in the Unite de la Tuberculose et des Mycobacteries, Institut Pasteur de Guadeloupe (http://www.pasteur-guadeloupe.fr/tb). By decoding the spoligotypes in the database, worldwide prevalent *M. tuberculosis* isolates have been classified into seven major families and some minors (Filliol et al., 2002). With more spoligotyping data available, novel families have been and will be defined (Garcia de Viedma et al., 2005). However, the discriminatory power of spoligotyping is generally poor, not able to provide sufficient discrimination between isolates, especially for the Beijing genotype strains, this method is not informative as vast majority of Beijing strains share an identical spoligotype (van Soolingen et al., 1995, Kremer et al., 2004).

1.3.3 MIRU-VNTR Typing

The genome of *M. tuberculosis* contains many minisatellite-like variable number tandem repeat (VNTR) loci (Frothingham and Meeker-O'Connell, 1998; Supply et al., 2000), some of these VNTR loci are named as mycobacterial interspersed repetitive unit (MIRU) loci due to some specific genetic features of the loci in mycobacteria (Supply et al., 2000). The repeat sequences of each locus are either identical or slightly variable in sequence or length (Frothingham and Meeker-O'Connell, 1998; Supply et al., 2000). MIRU-VNTR typing makes use of 12 such loci which are variable between strains in the

number of repeats; after PCR amplification of the 12 locus DNA sequences, the length of the PCR amplicons is converted into repeat numbers in term of every specific repeat length (Marzas et al., 2001).

Similarly as spoligotyping, MIRU-VNTR typing is a PCR-based typing method, therefore needs only 10^3 to 10^6 times less DNA than does IS*6110* RFLP, hence can provide 'real-time' typing results. But, this technique is highly reliable and the most reproducible, easy to perform, and MIRU-VNTR patterns are documented in 12 digital numbers. More importantly, MIRU-VNTR typing is comparable to IS*6110* RFLP typing in discriminatory power (Mazars et al., 2001), and performs greatly better than IS*6110* RFLP for strains with low IS*6110* copies, particularly for strains with one or zero IS*6110* copy (Mazars et al., 2001; Cowan et al., 2002). Moreover, this method can achieve substantially better discrimination for Beijing strains than does spoligotyping which is not discriminatory (Supply et al., 2001; Kam et al., 2005). MIRU-VNTR typing has been adapted high-throughput automation using gel-electrophoresis-based genescan analysis (Supply et al., 2001) which makes this method suitable for the global study of the molecular epidemiology of *M. tuberculosis*.

1.4 Epidemiological Applications of *M. tuberculosis* DNA Fingerprinting

The development of DNA fingerprinting techniques for typing *M. tuberculosis* isolates has led to an increasing number of studies of the molecular epidemiology of tuberculosis. The principal basis of molecular epidemiology of tuberculosis is to determine the genetic relatedness between clinical *M. tuberculosis* isolates by DNA fingerprints, and then use this to determine the clinical or epidemiological relationships of the isolates. Due to the

nature of clonal expansion of *M. tuberculosis*, epidemiologically-related isolates have identical or nearly identical DNA fingerprints, whereas epidemiologically-unrelated isolates show distinct DNA fingerprints from each other. Thus, the relationships between clinical isolates can be inferred from their DNA fingerprints (van Soolingen, 2001). In this application, however, the genetic marker used for strain-typing is pivotal. IS*6110* RFLP has been the most used on the basis of assumption that IS*6110* RFLP patterns in epidemiologically unrelated *M. tuberculosis* strains are sufficiently variable to label each strain as unique one, whereas epidemiologically related *M. tuberculosis* strains show identical or highly similar (one or two band difference) patterns. The validity of this assumption depends on the evolutionary speed of IS*6110*; it should be fast enough to generate substantial diversities of IS*6110* RFLP to distinguish unrelated strains yet stable enough in a certain time interval to identify isolates of the same strains in epidemiological events (Yeh et al., 1998). A number of studies have analyzed the stability of IS*6110* RFLP patterns of *M. tuberculosis* clinical isolates with > 5 IS*6110* copies and shown that IS*6110* insert is at a suitable evolutionary speed to be used in this connection (Cave et al., 1994; de Boer et al., 1999; Niemann et al., 2000; Warren et al., 2002a). For example, a study conducted in The Netherlands using 544 serial isolates from patients found that the half-life of IS*6110* RFLP patterns was 3.2 years (de Boer et al., 1999). This means that on average half of the stains exhibit a band shift in their IS*6110* RFLP patterns in a period of 3-4 years. This interval is sufficient for distinguishing epidemiologically-related and -unrelated isolates. This has been supported by many application studies in different settings over the years (Alland et al., 1994; Small et al., 1994; Borgdorff et al., 1998; van Soolingen et al., 1999; Garcia-Garcia et al., 2000a; van

Deutekom et al., 2004). In the study by van Soolingen et al. (1999), the authors found that 2 years may be a suitable study period to analyze transmission, shorter than that would underestimate transmission, longer than that would overestimate transmission.

However, IS*6110* RFLP is not suitable for isolates with ≤ 5 IS*6110* copies, especially for isolates with one or zero copies (Hermans et al., 1991; Yuen et al., 1993; Borgdorff et al., 1998). In this case, a secondary typing method, such as the polymorphic GC-rich sequence (PGRS) RFLP (van Soolingen et al., 1993; Borgdorff et al., 1998), spoligotyping (Goguet-de-la-Salmoiere et al., 1997; Bauer et al., 1999), and MIRU-VNTR typing (Mazars et al., 2001; Kwara et al., 2003), is needed to increase resolution. The PGRS RFLP typing is also a complicated method and difficult to be standardized, thus not be often used. Spoligotypes are too stable to yield satisfactory discrimination, often overestimate clustering rate; especially in the areas with high proportions of Beijing genotypes strains, spoligotyping is almost not informative whether used as first-line or secondary typing method (van Soolingen et al., 2001). MIRU-VNTR typing has been shown a suitable secondary typing method (Mazars et al., 2001; Cowan et al., 2002; Kwara et al., 2003; Blackwood et al., 2004), and some studies have suggested using MIRU-VNTR typing as first-line method either as alternative of IS*6110* RFLP typing (Blackwood et al., 2004) or in combination with IS*6110* RFLP typing (Mazars et al., 2001; Supply et al., 2001; Cowan et al., 2002; Kwara et al., 2003). On the other hand, some studies have also cast doubt to the suitability of MIRU-VNTR typing towards its application in molecular epidemiology because it can split clusters consisting of isolates with high number of IS*6110* copies (Kam et al., 2005; Scott et al., 2005). Therefore, more

studies based on confirmed epidemiological events are needed to further evaluate MIRU-VNTR typing in settings with different *M. tuberculosis* population structures.

1.4.1 Identification of Outbreaks and Transmission Analysis of *M. tuberculosis*

Tuberculosis is a disease spread by transmission from person to person. A major tuberculosis control measure is to interfere with the transmission of the bacilli by identifying foci of transmission. DNA fingerprinting has been used in many settings to define outbreaks and to estimate the extent of recent transmission (Alland et al., 1994; Small et al., 1994; van Soolingen et al., 1999; van Deutekom et al., 2004). In this regard, molecular epidemiological analysis of tuberculosis has proven markedly more effective than conventional epidemiological tools, which have very limited value in this situation (Small et al., 1994; van Deutekom et al., 2004). In the population-based study by Deutekom et al. (2004), as high as 86% of epidemiologically-related patients were not identified by conventional contact tracing.

1.4.2 Differentiation of Endogenous Reactivation and Exogenous Reinfection

Recurrent tuberculosis may result from the reactivation of endogenous primary infection (relapse) or from a recent exogenous reinfection (van Rie et al., 1999). DNA fingerprinting serves as a conclusive method to differentiate these two events from each other by fingerprinting *M. tuberculosis* isolates of the primary and recurrent episodes. If the paired isolates of primary and recurrent episodes of one patient are identical (or nearly identical, with one or two band difference) in their DNA fingerprints (usually IS*6110* RFLP), the recurrent event is regarded as a reactivation; otherwise, if the paired

isolates exhibit different DNA fingerprints, the recurrent event is considered to be reinfection. Studies in this connection have changed the traditional perspective that recurrent tuberculosis could be only a result of endogenous reactivation of primary infection. It is now considered that even in areas with low incidence of tuberculosis, reinfection could contribute to tuberculosis recurrence (Bandera et al., 2001; Garcia de Viedma et al., 2002). An accurate differentiation of reactivation and reinfection is essential for the determination of treatment failure rate and transmission level.

1.4.3 Identification of Laboratory Cross-contamination

Laboratory cross-contamination can lead to incorrect diagnosis and it has been reported in a prospective multicenter study that about 2% of all positive cultures are due to laboratory cross-contaminations (Jasmer et al., 2002). Therefore, it is important to identify cross-contamination as a regular practice. DNA fingerprinting has been used to identify or to confirm laboratory cross-contaminations. In this regard, IS*6110* RFLP typing seems more powerful due to its faster evolutionary speed (Small et al., 1993; Bauer et al., 1997); but a recent study has also demonstrated the utility of MIRU-VNTR typing in this aspect (Allix et al., 2004).

1.4.4 Identification of Simultaneous Infection with Multiple Strains

Simultaneous infections with multiple strains have been reported and documented by both IS*6110* RFLP typing (Yeh et al., 1999; Das et al., 2004) and MIRU-VNTR typing (Allix et al., 2004). Mixed infection could be confused with exogenous reinfection and laboratory cross-contamination.

1.5 Other Applications of DNA Fingerprinting of *M. tuberculosis*

1.5.1 Improving Speciation of *M. tuberculosis* Complex Isolates

DNA fingerprinting has led to improvements in identification and recognition of subspecies of the MTC that do not fit the previous classifications based on biochemical tests and growth characteristics. For example, in a study in Guinea-Bissau, Källenius et al. (Källenius et al. 1999) found that 140 out of 229 MTC strains could be allocated into one of three biovars, representing a spectrum between the classical bovine and human tubercle bacilli, using biochemical criteria. Although phenotypically heterogeneous these strains were genomically homogeneous and it was proposed that these strains constitute a distinct branch of the MTC tree between classical *M. bovis* and classical *M. tuberculosis* (Koivula et al., 2004) based on genetic markers. In another study by Niemann et al., (Niemann et al., 2002), the authors found that phenotypically-defined *M. africanum* subtype II is the main cause of human tuberculosis in Kampala, Uganda; by using genetic markers these strains has recently been reclassified as modern *M. tuberculosis* strains (Mostowy et al., 2004).

1.5.2 Uncovering of Population Structures of *M. tuberculosis*

Studying changes in population structure of *M. tuberculosis* is important to understand the adaptation of infectious agents to control measures. Strain-typing of isolates has revealed that the population structure of *M. tuberculosis* varies geographically. In low-incidence areas, IS*6110* RFLP patterns were highly polymorphic (Small et al., 1994; van Soolingen et al., 1999; Blackwood et al., 2005), reflecting the importance of reactivated disease, whereas the *M. tuberculosis* isolates in high-incidence areas showed much more

homogeneous IS*6110* RFLP patterns (Das et al., 1995; Bhanu et al., 2002), reflecting the active occurrence of ongoing transmission. DNA fingerprinting studies have also revealed that many local dominant clones of *M. tuberculosis* were endemic in different areas (van Soolingen et al., 1995; Bhanu et al. 2002; Douglas et al., 2003). This local dominance of specific *M. tuberculosis* clones suggests their selective advantages over others (van Soolingen et al., 1995), and may be related to local human biological and/or environmental factors (Hirsh et al., 2004).

1.5.3 Phylogenetic and Evolutionary Analysis

DNA fingerprinting generated huge amount of genetic data which have been used to study the phylogeny and evolution of the MTB members. Analyses by various genetic markers indicate that *M. tuberculosis* evolves and disseminates by clonal expansion (Warren et al., 2001; Supply et al., 2003; Baker et al., 2004) which results in great geographic variations in the distribution of *M. tuberculosis* evolutionary lineages (Sola et al., 2002; Filliol et al., 2002; Baker et al., 2004).

Spoligotyping is the most useful typing technique for phylogenetic study. Based on spoligotype, the global *M. tuberculosis* isolates can be well assigned into seven major evolutionary lineages and some minor ones, each family is defined by common family characteristics of spoligotype (Filliol et al., 2002; Sebban et al., 2002, Kremer et al., 2004). Grouping of *M. tuberculosis* isolates by spoligotype can reveal useful information for understanding of the evolutionary history, the phylogeographical distribution, the global transmission of the bacilli (Sola et al., 1999, 2001; Warren et al., 2002b; Dale et al., 2003; Filliol et al., 2003, Baker et al., 2004), and family-specific disease phenotypes

and pathogenesis (Glynn et al., 2002; Bifani et al., 2002, Baker et al., 2004). This application has been widely used to identify Beijing genotype strains for studying their relationships with various phenotypes (Anh et al., 2000; van Crevel., 2001; Lan et al., 2003; Toungoussova et al., 2003; Drobniewski et al., 2005).

1.6 Human Immunity to Tuberculosis

1.6.1 Innate Immunity

Whether an individual infected with *M. tuberculosis* does or does not develop clinical disease is determined by the complex immune interplay between host and the pathogen. It is estimated that in the infected population only 5-10% progress to active tuberculosis, 90-95% never develop active disease but remain lifelong asymptomatic latent infection; and among the diseased, about 85% of cases involve exclusively the lungs only (Boom et al., 2003; North and Jung, 2004).

It is generally believed that the initial immune defense to *M. tuberculosis* is the local innate immunity in lung, mediated primarily by alveolar macrophages. Inhaled mycobacteria are engulfed by alveolar macrophages through phagocytosis, and the macrophages can inhibit their growth and kill them via a variety of antimicrobial mechanisms. The degradation of phagocytosed mycobacteria by intralysosomal acidic hydrolases upon phagolysosome fusion constitutes a significant antimicrobial mechanism of phagocytes (Cohn and Wiener, 1963). Macrophages can kill mycobacteria through effector functions by producing reactive oxygen intermediates (ROI), such as H_2O_2 (Flesch and Kaufmann, 1987), and reactive nitrogen intermediates (RNI), such as nitric oxide (NO) and related RNI via inducible nitric oxide synthase (iNOS) (Nicholson et al,

1996; MacMicking et al., 1997). These effector functions are believed to be upon the activation of phagocytes by interferon-ϒ (IFN-ϒ) and tumor necrosis factor-α (TNF-α). Another potential mechanism involved in macrophage defense against *M. tuberculosis* is apoptosis of infected cells (Placido et al., 1997). This TNF-α mediated programmed cell death can limit outgrowth of *M. tuberculosis* (Placido et al., 1997), reduce viability of intracellular mycobacteria (Molloy et al., 1994).

In addition to lung macrophages, natural killer (NK) T lymphocytes also involve in host innate immunity against mycobacteria. T lymphocytes can be recruited to the macrophages and further stimulate it, possibly by producing IFN-ϒ (Iho et al., 1999), to inhibit growth of or kill mycobacteria. Cytotoxic T lymphocytes can ingest macrophages that have engulfed mycobacteria (Stenger et al., 1997) and kill them through apoptosis.

Toll-like receptors (TLRs) are phylogenetically conserved mediators of innate immunity which are essential for microbial recognition on macrophages and dendritic cells (Medzhitov and Janeway Jr., 1997). The importance of TLRs in tuberculosis immunity is that they can recognize the wall components of *M. tuberculosis*, such as lipoarabinomanan (LAM), and through this specific route *M. tuberculosis* can activate macrophages and dendritic cells. The specific TLRs so far identified for *M. tuberculosis* are TLR-2 and TLR-4 (Means et al., 1999).

But the fate of ingested bacilli in macrophages depends on the virulence of individual mycobacterial isolates and the intrinsic microbicidal capacity of host phagocytes. A recent study has shown the substantial variability in the capacity of clinical tuberculosis isolates to replicate in host cells in the face of innate host immunity (Janulionis et al., 2005). The importance of the intrinsic microbicidal capacity of host

phagocytes has been shown by the associations between the polymorphisms of some human genes which encode various macrophage products, such as the natural resistance-associated macrophage protein (NRAMP1) gene, the interleukin 1 (IL-1) gene cluster, the vitamin D receptor gene and mannose-binding lectin gene, and the susceptibility to *M. tuberculosis* (Bellamy et al., 2000). Individuals with certain polymorphisms in these genes may render them susceptible to mycobacterial infection. However, to what extent these genes can affect the susceptibility is unknown. A case-control study on vitamin D deficiency in the Gujarati population in London showed that such effect is likely small (Wilkinson et al., 2000).

1.6.2 Acquired Immunity

If innate immune responses fail to eliminate ingested bacilli, the surviving bacilli will multiply and stimulate the immune system to develop adaptive immunity. In fact, the ubiquitous acquisition of anti-tuberculosis specific adaptive immunity in the diseased and the latently infected suggest that the innate cellular immunity often fail to eliminate the bacteria.

Humoral immunity. It is generally assumed that acquired humoral immune response is not relevant with protection in tuberculosis, but maybe there are some antibodies are protective (Teitelbaum et al., 1998).

Cellular immunity. Cell-mediated immune responses are pivotal in tuberculosis, which can be protective or detrimental. The protective immunity against tuberculosis is called Th1 immune response, which is characterized by Th1 cytokines, primarily IFN-ϒ, IL-2, and IL-12. The detrimental immunity in tuberculosis is called Th2 immune response,

which is characterized by Th2 cytokines, mainly IL-4, IL-5, and IL-13 (reviewed by Rook et al., 2001).

IFN-ϒ. The role IFN-ϒ for a protective immunity in tuberculosis has been well established. In children who have genetic defects in IFN-γ receptor gene which result in deficiency of IFN-γ receptor, BCG vaccination, which is widely used to protect tuberculosis infection, can cause severe disseminated disease (Jouanguy et al., 1996; Newport et al., 1996). In gene knock-out (KO) mice incapable of making IFN-γ fail to acquire the ability to inhibit *M. tuberculosis* growth in their lungs and other organs (Cooper et al., 1993; Flynn et al., 1993). *in vitro* IFN-γ production upon mycobacterial antigen-specific stimulation has been substantially investigated and used as a surrogate diagnostic marker for *M. tuberculosis* infection (van Crevel et al., 1999).

IL-2. IL-2 can induce lymphocyte expansion in the context of antigen-specific stimulation. It has been demonstrated that IL-2 can influence the course of mycobacterial infection either alone or in combination with other cytokines (Blanchard et al., 1989).

IL-12. The central role of IL-12 is to induce the production of IFN-γ. It is a key player in host defense against *M. tuberculosis* in both innate and adaptive immunity. IL-12 KO mice are highly susceptible to mycobacterial infections (Cooper et al., 1997). In humans who have genetic defects in IL-12 receptor gene and contracted mycobacterial infections, the effect of IL-12 receptor deficiency is due to the significant reduced production of IFN-γ by NK cells and T cells which is induced by IL-12 (Altare et al., 1998; de Jong et al., 1998). This further emphasizes the importance of IFN-γ.

IL-18. In addition to IL-12, IL-18 is another important IFN-γ-inducing cytokine (O'Neill and Greene, 1998), and it can stimulate the production of other proinflammatory

cytokines (Netea et al., 2000b). Synergistically IL-12 and IL-18 strongly favor development of Th1 cytokine responses.

IL-4. IL-4 is a major Th2 cytokine which can suppress IFN-γ production and macrophage activation (van Crevel et al., 2002), and switch of signaling via TLR-2 and potently down regulate iNOS (Bogdan et al., 1994). In mice infected with *M. tuberculosis*, progressive disease and reactivation of latent infection are both associated with increased production of IL-4 (Hernandez-Pando et al., 1996; Howard and Zwilling, 1999), and overexpression of IL-4 intensified tissue damage in experimental infection (Lukacs et al., 1997). In humans, overproduction of IL-4 has been associated with more extensive radiological disease (Seah et al., 2000), with cavitary tuberculosis (van Crevel et al., 2000), and with progression from latent infection to active disease (Ordway et al., 2004). All these findings suggest that IL-4 may be a major pathogenic factor in tuberculosis (Rook et al., 2005a). The increased production of IL-4 has been thought to result in the imbalance of Th1 and Th2 cytokines, and this imbalance may play a major role in the pathogenesis of tuberculosis (Howard and Zwilling, 1999; Barnes and Wizel, 2000).

TNF-α. TNF-α is a prototype proinflammatory cytokine which plays a key role in granuloma formation (Kindler et al., 1989; Senaldi et al., 1996), activates macrophage inhibiting growth of intracellular mycobacteria (Barnes and Modlin, 1996). On the other hand, TNF-α also contributes to immunopathology of tuberculosis by participating in host-mediated destruction of lung tissue. The switch from protective to deleterious role of TNF-α is believed to be associated with IL-4 (Rook et al., 2005a).

T-cell subtypes involved in adaptive immunity. Studies in humans and animals demonstrate that adaptive immunity to *M. tuberculosis* requires contributions by multiple

T cell subsets, which include α/β CD4+ and CD8+ cells, ϒ/δ T cells, and CD1-restricted T cells. These cells can only control or maintain the infection, cannot eradicate the bacteria (reviewed by Boom et al., 2003).

α/β CD4+ T cells. This subset of T lymphocytes is the central player in acquired tuberculosis immunity. This can be well reflected by HIV positive individuals. HIV positive persons, who have defective CD4+ cellular immunity, are at markedly increased risk to contract tuberculosis either from new infection or from reactivation of latent infection (Corbett et al., 2003). Mice with CD4+ T cell deficiency are greatly susceptible to *M. tuberculosis* (Caruso et al., 1999), and in a murine model of chronic persistent *M. tuberculosis* infection, CD4+ T cell depletion caused rapid reactivation of the infection (Scanga et al., 2000).

The primary effector function of CD4+ T cells is the production of IFN-γ and TNF-α, essential cytokines to activate macrophages. *M. tuberculosis* antigen activated CD4+ T cells are cytotoxic to macrophages infected by *M. tuberculosis* and help macrophages control intracellular mycobacteria (Boom et al., 2003).

α/β CD8+ T cells. CD8+ T cells can secret IFN-γ and IL-4 and thus may play a role in regulating the balance of Th1 and Th2 immunity. Increased production of IL-4 by CD8+ cells and ϒ/δ T cells is associated with progression from latent infection to active disease in health-care workers (Ordway et al., 2004). *M. tuberculosis* antigen activated CD8+ cells can lyse *M. tuberculosis*-primed macrophages and thus help macrophages to control the infection (de Libero et al., 1988).

ϒ/δ T cells. ϒ/δ T cells are cytotoxic, can kill *M. tuberculosis*-infected macrophages and reduce the viability of intracellular bacteria (Rook et al., 2001). ϒ/δ T cells selectively

expand when stimulated *in vitro* by live *M. tuberculosis* (Barnes and Modlin, 1996). In addition, ϒ/δ T cells from tuberculin-negative individuals and from newborns proliferate in response to *M. tuberculosis*, suggesting they also participate in innate immunity (Barnes and Modlin 1996).

1.7 The Beijing Genotype of *M. tuberculosis*

1.7.1 Definition of Beijing Genotype Strains

In 1995, van Soolingen *et al*. identified a group of genetically highly conserved *M. tuberculosis* strains from the Beijing area of China. In the patterns of IS*6110,* IS*1081*, and the polymorphic GC-rich repeat sequence (PGRS) RFLP, these strains were distinct from then-known *M. tuberculosis* strains but closely related within the group; moreover, in the direct repeat (DR) region, all the strains exhibited an identical spoligotype that in the 43 spacer format of spoligotype had spacers 1 to 34 deleted and 35-43 conserved. Because this group of *M. tuberculosis* strains were first discovered and highly prevalent (> 85%) in the Beijing area, strains in this group were designated the Beijing genotype (van Soolingen *et al*., 1995).

With more global genotyping data available, it is found that the identical spoligotype is specific to the Beijing genotype strains. It is also found however that the nine spacers 35 to 43 are not invariably present in all Beijing strains, some Beijing-like spoligotypes (lacking one or more of the last 9 spacers in addition to spacers 1 to 34) were uncovered in some areas of the world (Diaz et al., 1998; Chan et al., 2001; van Crevel et al., 2001; Kremer et al., 2004). Kremer *et al*. (2004) have recently characterized the strains with Beijing-like spoligotypes and proven that they belong to the Beijing

genotype; a new definition for the Beijing genotype strains thereby has been recommended which defines Beijing strains as strains hybridizing to at least three of the nine spacers 35 to 43 and with absence of hybridization to spacers 1 to 34 by spoligotyping. Those which hybridize with all the nine spacers of 35 to 43 are termed "typical Beijing strains", otherwise, called "atypical Beijing strains". By this definition, spoligotyping can serve as a "gold standard" method to identify Beijing lineage strains (Kremer et al., 2004).

The W strain (Bifani et al., 1996), a MDR clone associated primarily with institutional outbreaks in New York City (Valway et al., 1994; Frieden et al., 1996; Moss et al., 1997), is a variant of Beijing family strains (Bifani et al., 1999). Therefore, the Beijing genotype is also known variously as Beijing\W or W-Beijing in some studies (Bifani et al., 2002; Glynn et al., 2002; Kremer et al., 2004).

1.7.2 Global Dissemination of the Beijing Genotype Strains

In addition to the Beijing area, the *M. tuberculosis* Beijing genotype strains were found to be also predominant in other Asian areas, such as Mongolia (van Soolingen et al., 1995), Korea (Park et al., 2000), Vietnam (Anh et al., 2000), Hong Kong (Chan et al., 2001), and in Russia (Pfyffer et al., 2001; Drobniewski et al., 2002; Toungoussova et al., 2002, 2003), and highly prevalent in some states of the United States (Yang et al., 1998; Bifani et al., 1999, 2001; Soini et al., 2000), Thailand (Prodinger et al., 2001), Malaysia (Dale et al., 1999), Indonesia (van Crevel et al., 2001). In 2002, Bifani et al. and Glynn et al. independently made systematic reviews of published papers in which Beijing strains could be identified based on different genetic markers, it was showed that Beijing strains

are widely distributed worldwide (Bifani et al., 2002; Glynn et al., 2002). The reason for the selective expansion of the Beijing genotype strains over other genotype strains has been speculated as that Beijing strains may be able to escape from the protection of BCG vaccination but other genotype strains are inhibited by the protection of BCG vaccination (van Soolingen et al., 1995). Limited supportive data for this hypothesis have been obtained from an animal study in which pre BCG vaccination seemed to inhibit the multiplication of *M. canettii* and *M. tuberculosis* laboratory strain H37Rv but not Beijing strains (López et al., 2003). However, no supportive evidence for this hypothesis has been obtained from molecular epidemiological studies (Bifani et al., 2002; Glynn et al., 2002).

Beijing strains have been found to be more likely involved in DNA fingerprint defined cluster (Toungoussova et al., 2002, 2003) which is generally assumed as a result of recent active transmission (Small et al., 1994; Glynn et al., 1999). In addition to the higher rate of clustering, Beijing strains were also found to spread rapidly in a community from 5.5% to 27% in 3 years (Caminero et al., 2001). This suggests that Beijing strains are highly transmissible.

1.7.3 Clinical and Epidemiological Phenotypes of Tuberculosis Associated with the Beijing Genotype

In addition to the wide dissemination, Beijing strains have been frequently reported to be associated with drug-resistance in Vietnam (Anh et al., 2000), New York (Bifani et al., 1996), Cuba (Diaz et al., 1998), Estonia (Kruuner et al., 2001), and some areas of Russia (Pfyffer et al., 2001; Drobniewski et al., 2002; Toungoussova et al., 2002, 2003).

Whereas in other areas, such as Hong Kong (Chan et al., 2001), Thailand (Prodinger et al., 2001), and Indonesia (van Crevel et al., 2001), this association was not observed. In Vietnam, Beijing strains were also found to be associated with younger patients (Anh et al., 2000) but this was not demonstrated in other areas (Glynn et al., 2002). Therefore, the relationship of the Beijing genotype with drug-resistance and with patient's age remains a matter of debate. In addition, also in Vietnam the Beijing genotype was found to be associated with treatment failure and tuberculosis relapse (Lan et al., 2003), and in Indonesia it was associated with febrile response to treatment (van Crevel et al., 2001). Very recently, Warren and colleagues have found that Beijing strains are involved in most of multiple infections in a single episode (Warren et al., 2004).

1.7.4 Potential Virulent Genetic Factors of the Beijing Genotype

The bacterial factor(s) that is responsible for the differential epidemiological and clinical phenotypes of tuberculosis remains largely unknown. One potential genetic factor is the pks1-15gene encoding polyketide synthase-derived phenolic glycolipid (PGL), which confers Beijing strains with the ability to inhibit the release of key inflammatory effector molecules by the cells of the host's innate immune response (Reed et al., 2004). The pks1-15 gene is unable to encode PGL in non-Beijing strains due to a frameshift mutation resulting from 7-base-pair deletion of the gene, but Beijing strains have an intact pks1-15 gene, thereby being able to produce PGL. The mobile element IS*6110* is another possible virulent genetic factor. Most of Beijing genotype strains contain high numbers of IS*6110* copy compared to non-Beijing strains, and all Beijing strains share IS*6110* insertion sites. One of them is in the bacterial chromosomal origin of replication, and many of them are

within coding regions which result in disruption of the coding genes (Bifani et al., 2002). A recent study has shown that IS*6110* can up-regulate downstream genes through an outward-directed promoter in its 3' end which may alter the expression of adjacent genes to favor the specific phenotypes (Safi et al., 2004). The whole genome sequencing of *M. tuberculosis* Beijing strain 210 is in progress in The Institute for Genomic Research (TIGR, http://www.tigr.org). The availability of this whole genomic sequence will shed more light into the relationship between genomic structure of Beijing strains and the phenotypes.

1.7.5 Specific Immunological Pathogenesis of the Beijing Genotype Strains

The associations of Beijing genotype strains with the specific phenotypes of tuberculosis have attracted increasing research attention to virulence of Beijing genotype and immune response against this genotype strains. In an interesting study (López et al., 2003) which compared immune response, pathogenesis and BCG protection in mouse model of pulmonary tuberculosis induced by four genotypes of *M. tuberculosis* strains, Beijing strains were characterized by significantly higher bacillary burdens, extensive pneumonia, and significantly higher and earlier mortality. Previous BCG vaccination protects less effectively against infection with Beijing strains than against the laboratory strain H37Rv. However, the patterns of cytokine secretion are intriguing. INF-γ gene expression in lungs of mice infected with Beijing strains is delayed and remarkably weaker than those infected with laboratory strain H37Rv, showing some correlation with bacterial loads and disease phenotypes. In contrast, although *M. canettii* strains induce

similar INF-γ gene expression level as Beijing strains, the disease phenotypes, particularly mortality, between the two genotypes differ markedly.

Similarly, in another animal study which characterized a Beijing strain, HN878, the high early death of infected mice with HN878 was associated with failure to induce INF-γ production by spleen and lymph node cells from infected mice. Moreover, the expression of INF-γ, TNF-α, IL-6 and IL-12 were significantly lower in lungs of HN878-infected mice (Manca et al., 2001). This is in contrast with an outbreak strain CDC1551, a well characterized non-Beijing strain, which induces earlier and strikingly higher level expressions of INF-γ, TNF-α, IL-6 and IL-12 genes in lungs of mice infected with CDC1551 which are associated with long term survival of the mice (Manca et al., 1999). Furthermore, in humans, infection with CDC1551 also induced strong immune responses characterized by very large skin test responses to purified protein derivative of tuberculin (PPD) and more latent infections than active diseases (Valway et al., 1998).

Although CDC1551 grew much faster than laboratory strain Erdman in lungs of mice, this was not found when compared to strains HN878 and H37Rv. In addition, the growth rates of CDC1551 and H37Rv in human monocytes were also the same. However, another outbreak strain, strain 210 (a Beijing family member), was found to grow markedly faster than CDC1551 and other clinical isolates but they all produced similar amounts of TNF-α, IL-6, IL-10, and IL-12, and were equally susceptible to reactive nitrogen intermediates in human macrophages (Zhang et al., 1999).

1.8 Molecular Epidemiology of Drug-resistant Tuberculosis

1.8.1 Types of Drug Resistance

The phenomenon of resistance of *M. tuberculosis* to anti-tuberculosis drugs was observed shortly after the introduction of effective antituberculosis agents into clinical practice in 50 years ago (Crofton and Mitchison, 1948; Szyblaski and Bryson, 1952), but only received international attention in the early 1990s when drug-resistant *M. tuberculosis* strains were frequently found to be involved in outbreaks in patients with HIV infection in the United States and Europe (Pablos-Mendez et al., 1998).

Drug resistance of *M. tuberculosis* is classified as primary or acquired. When resistance occurs in a patient who has never undertaken anti-tuberculosis therapy previously, it is termed primary resistance. The acquisition of primary resistance results from the transmission of drug-resistant *M. tuberculosis* isolate from a patient to another person. The level of primary resistance in a community is therefore an indicator of transmission within the community. Acquired resistance is that which occurs as a result of unsuccessful anti-tuberculosis treatment due to a number of man-made factors, such as improper medical management, inappropriate prescription, or poor adherence of patients to treatment, *etc*.. The level of acquired resistance reflects the performance of on-going tuberculosis control program (WHO, 1997, 2000b). However, the two proxies "resistance among new cases" and "resistance among previously treated cases" have now been adopted to replace the terms acquired and primary resistance respectively (WHO, 2000b; Espinal et al., 2001).

1.8.2 Burden of Drug-resistant Tuberculosis

Being launched in 1994, the global project of resistance surveillance to antituberculosis drugs conducted by WHO/IUATLD has obtained considerable information on the global

burden and trends of drug-resistant tuberculosis over the last 10 years (WHO, 1997, 2000b, 2004; Pablos-Mendez et al., 1998; Espinal et al., 2001). The incidences vary significantly between geographic settings. The very recent WHO report showed that the prevalence rates ranged from the lowest 0% in some Western European countries to the highest 57.1% (median = 10.2%) in Kazakhstan among new cases, and a wider range from 0% to 82.1% (again in Kazakhstan) among previously treated cases, with a median 18.4%; the higher prevalence rates were seen in Central Asia and some provinces of China and Russian Federation. With respect to prevalence trends, data from settings with at least two observation points showed significant increase in Botswana, New Zealand, Poland, and Tomsk Oblast of Russian Federation; on the other hand, significant deceases in prevalence were also observed in quite number of settings implementing tuberculosis control according to international guidelines over the years (WHO, 2004).

Overall, drug-resistant tuberculosis continues to be a serious threat to global tuberculosis control. Multidrug-resistant tuberculosis (MDR-TB), defined as resistance to at least the two most potent anti-tuberculosis drugs, i.e., isoniazid (INH) and rifampin (RIF), is a major threat since MDR-TB is significantly more difficult and expensive to treat than is drug-susceptible tuberculosis, and causes higher treatment failure, relapse and mortality (Mahmoudi and Iseman, 1993; Espinal et al., 2000; Garcia-Garcia et al., 2000a, 2000b; Quy et al., 2003). Although globally MDR-TB is low (median 1.1%), but in many specific regions of the world it is at levels of incidence greater than 10% (WHO, 2004; Espinal, 2003; Maher and Raviglione, 2005). MDR-TB may spread from those areas with high incidence to other areas with low incidence. Other resistance to antituberculosis drugs also possesses threat to tuberculosis control though maybe at a less

extent than does MDR. Streptomycin resistance, isoniazid resistance or polyresistance (resistance to more than two drugs but not MDR) has been found to be risk factors secondary to MDR for treatment failure or relapse in Vietnam (Quy et al, 2003). In addition, drug-resistant tuberculosis is usually to be infectious for a longer period than is drug-susceptible tuberculosis.

1.8.3 Transmission of Drug-resistant Tuberculosis

Molecular epidemiological studies in New York City showed that resistance to any drug (Alland et al., 1994) or MDR (Friedman et al., 1995) were important risk factors for patients being in a cluster. Similar finding was obtained from a study performed in Poland that patient with MDR disease was more likely to cluster (Sajduda et al., 2004). However, in Mexico (Garcia-Garcia et al., 2000a, 2000b) and in South African gold-mining community (Godfrey-Faussett et al., 2000), patients with MDR tuberculosis were significantly associated with a decreased likelihood of being clustered. Similarly, in San Francisco, patients with MDR tuberculosis were not likely to produce secondary drug-resistant cases (Burgos et al., 2003). In addition, in the Netherlands, van Soolingen et al. (1999) have found that INH-resistant strains were less likely to be in a cluster. Corroborating with this, Burgos et al. (2003) found that even clustered strains that were resistant to isoniazid alone or in combination with other drugs were less likely to result in secondary cases than were drug-susceptible strains. The discrepancy among these studies may be due to different population structures of *M. tuberculosis* in these areas because different genotypes of *M. tuberculosis* may have different propensities to develop drug-

resistance as reviewed in section 1.8.4, or maybe the studies in New York City involved in more high virulent drug-resistant strains.

1.8.4 *M. tuberculosis* Genotypes and Drug-resistant Phenotypes

The genetic basis of drug-resistance in *M. tuberculosis* is the resistance-conferring mutations in variety of genes in relation to antituberculosis agents (Ramaswamy and Musser, 1998). Spontaneous mutations leading to drug resistance occur rarely in the bacilli, man-made factors are responsible for the occurrence of drug-resistant mutants. However, molecular epidemiological studies in recent years have revealed increasing evidence that some drug-resistant patterns are not equally occurred among the family members of *M. tuberculosis* (Anh et al., 2000; Chan et al., 2001; Toungoussova et al., 2002, 2003; Baker et al., 2004; Hillemann et al. 2005; Jou et al., 2005). In Vietnam, resistance to isoniazid or streptomycin was found to more likely occur in Beijing genotype strains (Anh et al., 2000). In Russia, resistance to streptomycin, rifampin, or ethambutol but not to isoniazid was found to be associated with Beijing strains (Toungoussova et al., 2002, 2003). In the UK, resistance to streptomycin was associated with both Beijing and Delhi strains, but resistance to rifampin was not associated with any genotype (Baker et al., 2004). An association between resistance to isoniazid or ethambutol and Beijing genotype was also demonstrated in Taiwan (Jou et al., 2005). However, resistance to isoniazid was inversely associated with Beijing genotype in Hong Kong (Chan et al., 2001).

Moreover, by correlating resistance-conferring mutations with the genotypic lineages of *M. tuberculosis*, Baker et al. (2004) found that the streptomycin resistance-

conferring mutation *rpsL*^{A128G} was positively associated with Beijing strains, but inversely associated with Haarlem strains; the isoniazid resistance-conferring mutation *katG* ^{A944C} was positively associated with Delhi strains, and the *inhA* ^{C15T} promoter mutation positively associated with East-African-Indian (EAI) strains. In a very recent study in Germany, Hillemann et al. (2005) found that the isoniazid resistance-conferring mutation *katG* ^{S315T} and the rifampin resistance-conferring mutation *rpoB* ^{S531L} were associated with Beijing strains. These findings suggest that the resistance-conferring mutations in *M. tuberculosis* may not independently and randomly occur in different genotype strains but genotype-related, further suggesting possible role of intrinsic bacterial factors of the bacillium in the development of the resistance-conferring mutations.

1.9 Tuberculosis in Singapore

Singapore is a small island situated on the southernmost tip of the Malaysian peninsular. During the past few decades, Singapore has become industrialized and developed as a globalized city state. The overall incidence of tuberculosis in Singapore (per 100,000 population) has declined from > 200 in 1960s to about 100 in early 1980s and further to about 50 in 1990s (Figure 1.1). The incidence has reached its lowest ever rate at 39 in 2004 (Ministry of Health Singapore, 2005). However, the remarkable decrease in tuberculosis incidence is mainly due to the rapid increase in size of the Singaporean population, whereas the absolute number of tuberculosis cases notified did not change much over the period of 1987 to 2004 (Figure 1.2; data from Communicable Disease

Surveillance Report 2004, the Department of Clinical Epidemiology, Communicable Disease Centre, Tan Tock Seng Hospital and Ministry of Health).

In 1997, Singapore embarked the Singapore Tuberculosis Elimination Program (STEP) (Chee and James, 2003). The key component of the STEP is early diagnosis and proper treatment of patients with tuberculosis. This can cure and prevent further transmission of the disease and emergence of drug-resistant tuberculosis. The concerted efforts carried out under the STEP and by the medical community have contributed to the recent decline in tuberculosis cases in Singapore.

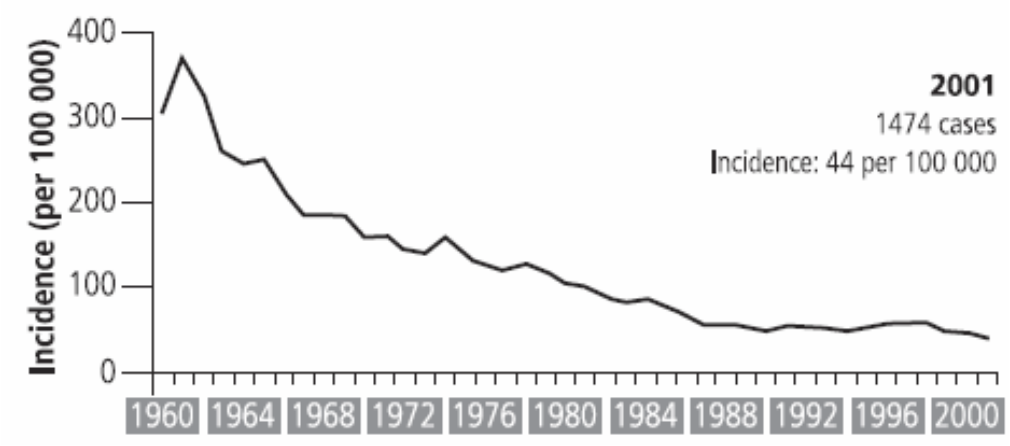

Fig. 1.1. Incidence of tuberculosis among Singapore residents, 1960-2001. (source: Chee and James, 2003).

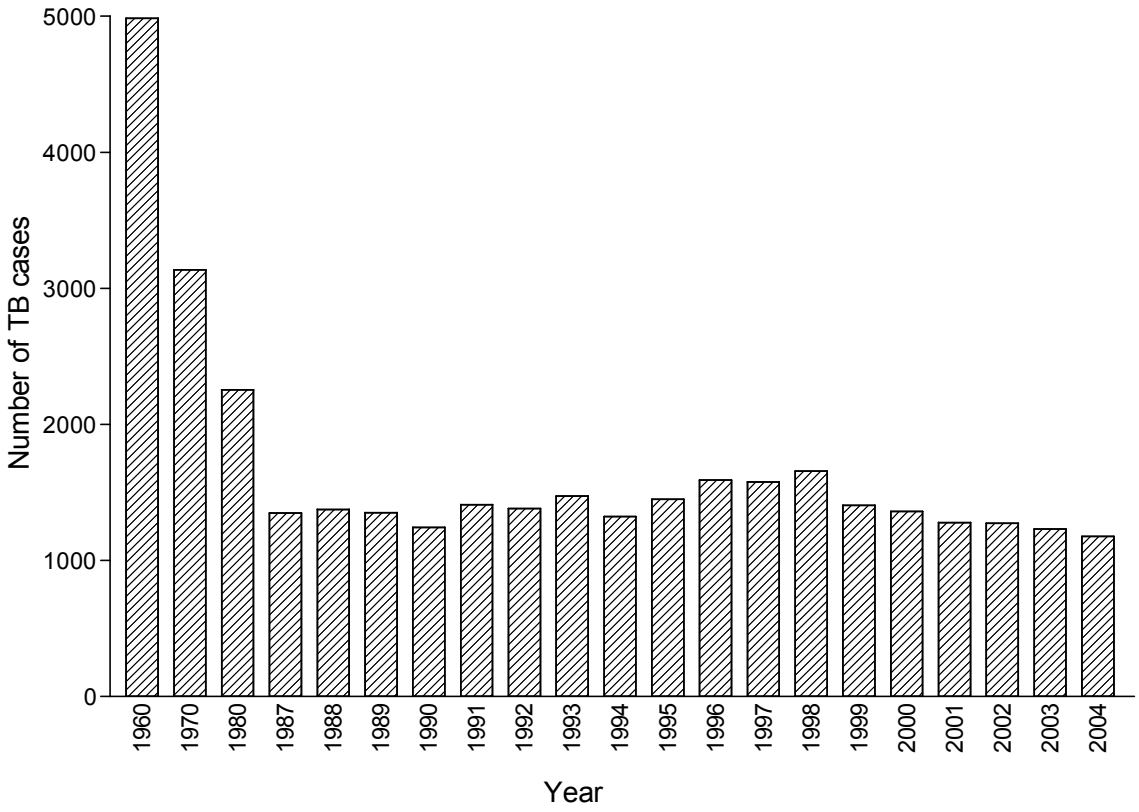

Fig. 1.2. Total number of tuberculosis cases notified among Singapore residents, 1987-2004.

Drug-resistant tuberculosis has been a persistent problem with an incidence in local residents fluctuating between 4.2% to 6.7% in the last decade (Communicable Disease Surveillance Report 2004, the Department of Clinical Epidemiology, Communicable Disease Centre, Tan Tock Seng Hospital and Ministry of Health). Lee and colleagues have done a series of studies (Lee et al., 1999, 2001, 2005) to examine drug-resistant mechanisms of *M. tuberculosis* in Singapore. However, little is known to date on the genetic diversity and molecular epidemiology of *M. tuberculosis* in this urban country.

1.10 Aims of the Present Project

In this PhD program, we designed several retrospective and prospective studies aimed to study the molecular epidemiology of tuberculosis in Singapore and to investigate clinical and immunological characteristics of tuberculosis associated with the *M. tuberculosis* Beijing and non-Beijing family strains. Our specific aims were:

1. To uncover the genetic diversity and population structure of *M. tuberculosis* in Singapore using three major genotyping methods, IS*6110* RFLP typing, spoligotyping, and MIRU-VNTR typing, as well as other minor typing methods.

2. To evaluate and compare the performances of the three major strain-typing methods and to formulate a genotyping proposal based on the local population structure of *M. tuberculosis* and the performances of the three major typing modalities.

3. To examine the relationship between Beijing strains and tuberculosis relapse in Singaporean patients.

4. To study the transmission dynamics of drug-resistant tuberculosis and relationships between genotypes and drug-resistant phenotypes of *M. tuberculosis*.

5. To clinically and immunologically characterize the tuberculosis associated with the Beijing and non-Beijing families of *M. tuberculosis*.

CHAPTER 2

GENETIC DIVERSITY AND GENOTYPING STRATEGY OF *M. TUBERCULOSIS*

2.1 Introduction

Tuberculosis remains a leading cause of death due to bacterial infection, with approximately 8 million new cases and 2 million deaths each year worldwide. It is estimated that one third of the world's population is infected with *M. tuberculosis*, the causative agent of tuberculosis (Corbett et al., 2003). Singapore has moderate tuberculosis incidence (40 per 100,000) in local residents (citizens and permanent residents). But an increasing burden has come from foreigners who work and study in this country. The effort to control the epidemic has been so far clinically oriented (Chee and James, 2003).

Molecular epidemiology of tuberculosis has been widely used in developed countries to study and to trace tuberculosis transmission (van Soolingen et al., 2003). It has been shown that molecular epidemiology is greatly more effective than is conventional contact tracing (van Deutekom et al., 2004). DNA fingerprinting of *M. tuberculosis* has gained increased acceptance as a useful tool for molecular epidemiological investigations (Alland et al., 1994; Small et al., 1994; Borgdorff et al., 1998; van Soolingen et al., 1999; Garcia-Garcia et al., 2000a; van Deutekom et al., 2004). During the past 15 years, a large number of DNA fingerprinting methods based on various genetic markers have been developed (van Soolingen, 2001; Mazars et al., 2001). As no single method however has defined all unique isolates so far, multistep typing strategies are needed for a better performance (Alland et al., 1994; Bauer et al., 1999; Yang et al., 2001; Cowan et al., 2002; Kwara et al., 2003).

The purposes of the present study were to discover the genetic diversity and population structure of *M. tuberculosis* in Singapore, to compare the novel MIRU-VNTR

typing with the more established spoligotyping and IS*6110* RFLP typing, and finally to define an optimal typing strategy for molecular epidemiological analysis of tuberculosis in Singapore.

2.2 Materials and Methods

2.2.1 Mycobacterial DNA samples

We used a bank of mycobacterial DNA samples that had been extracted from drug-susceptible *M. tuberculosis* clinical isolates consecutively collected between August and December 1994 from the Central Tuberculosis Laboratory, Singapore General Hospital. Isolates from 10 residential postal districts were collected and analyzed. The DNA samples were extracted using a standard protocol that is described elsewhere (van Soolingen et al., 1991). Briefly, mycobacterial culture was resuspended in 400 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH8.0) in a microcentrifuge tube and heated at 80^0C for 30 minutes to kill the bacteria. Lysozyme was added to a final concentration of 1 mg/ml, and the tube was incubated for 1 h at 37 ^{0}C. Followed by adding 70 µl of 10% SDS and 6 µl of proteinase K at concentration of 10 mg/ml and incubated for 10 min at 65^0C. After adding 80 µl of *N*-acetyl-*N,N,N*-trimethyl ammonium bromide (CTAB) and votexing briefly, the tube was incubated for 10 minutes at 65^0C. An equal volume of chloroform/isoamyl alcohol was added, and the mixture was votexed for at least 10 seconds. After centrifugation at 14,000 rpm for 5 minutes, 0.6 volume of isopropanol was added to the supernatant to precipitate DNA. After 30-minute incubation at -20^0C, DNA was pelleted by centrifugation at 14,000 rpm for 15 minutes, and washed with 70%

ethanol. The final DNA pellet was air-dried and dissolved in 20 µl of TE buffer. Samples were kept in -20^{0}C freezer until the current analysis.

2.2.2 Spoligotyping

A detailed spoligotyping protocol has been previously described (Kamerbeek et al., 1997). In this study, we used a commercial spoligotyping kit (Isogen Bioscience BV, The Netherlands), and the manufacturer's protocol was followed.

Spoligotyping PCR. Primers DRa (5'-GGT TTT GGG TCT GAC GAC-3') and DRb (5'-CCG AGA GGG GAC GGA AAC-3) flanking the DR region were used for spoligotyping PCR. Primer DRa is biotinylated at 5' end. PCR was performed using microwell PCR plate in 50 µl reaction volume mixture which contained 20 pmol of each of primer (DRa and DRb), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 1.5 mM of $MgCl_2$, 1x Q solution, 1x PCR buffer, 1 U of HotStartTaq DNA polymerase (Qiagen, Germany), and approximately 5 to 50 ng of DNA. The PCR was carried out using a PCR thermocycler (Biometra, Austria) starting with a denaturing step of 3 minutes at 96°C, followed by 40 cycles of 1 minute at 96°C, 1 minute at 55°C, and 30 seconds at 72°C. The reactions were terminated by incubation for 5 minutes at 72°C. Positive controls using *M. tuberculosi*s strain H37Rv and *M. bovis* BCG P3 and negative control using water were performed in parallel.

Hybridization and membrane development. PCR products were diluted by mixing 20 µl of PCR product and 150 µl 2x SSPE/0.1% SDS. The diluted PCR products were denatured at 99°C for 10 min and then immediately cooled on ice. The PCR products were added to a miniblotter which sandwiched a membrane with 43 synthetic oligonucleotides immorbilized in parallel rows. The 43 oligonucleotides represent the

unique variable spacer sequences between DR units in the DR region. After incubated at 60°C for 60 min, samples were removed from the miniblotter and the membrane was washed twice in 250 ml 2xSSPE/0.5% SDS for 10 minutes at 60°C. The membrane was then placed in a rolling bottle and incubated with 2.5 µl streptavidin-peroxidase (Amersham Bioscience, Sweden) in 10 ml 2xSSPE/0.5% SDS for 60 minutes at 42°C in a hybridization oven (Hybaid, UK). After the incubation, the membrane was washed twice in 250 ml 2xSSPE/0.5% SDS for 10 minutes at 42°C and subjected to chemiluminescence development using the ECL chemiluminescent detection kit for labeled nuclei acids. The membrane was exposed to a light sensitive ECL Hyperfilm (Amersham Bioscience) for 40 minutes and then developed using Kodak X-ray film developer and fixer. This generated a characteristic spoligotype pattern for each isolate, where a positive hybridization signal was indicative of the presence of a particular spacer.

2.2.3 MIRU-VNTR typing using genescan analysis

MIRU-VNTR PCR. This PCR has been previously described (Supply et al., 2001). The fluorescent-labeled PCR primers flanking each polymorphic MIRU-VNTR locus and multiplex mixtures are shown in Table 2.1. Each MIRU-VNTR locus was individually amplified using a 50 µl reaction volume in 96-well PCR plates. The reaction mixture contained 0.4 µM each of primer, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 1.5 mM of $MgCl_2$, 1x Q solution, 1x PCR buffer, 1 U of HotStartTaq DNA polymerase (Qiagen, Hilden, Germany), and approximately 5 to 50 ng of DNA. The thermocycler programs for the four multiplex reactions were identical. The PCRs were carried out using a PCR thermocycler (Biometra, Austria) starting with a denaturing step of

15 minutes at 95°C, followed by 40 cycles of 1 minute at 94°C, 1 minute at 59°C, and 1 minute 30 seconds at 72°C. The reactions were terminated by incubation for 10 minutes at 72°C. Negative controls consisted of the PCR performed on reaction mixtures lacking mycobacterial DNA. PCR products were appropriately diluted and pooled together by each multiplex mixture for genescaning. However, those samples that were not properly analyzed in the pooled sets were then diluted for individual capillary electrophoresis.

Table 2.1. Primers and conditions for multiplex PCRs of the 12 MIRU-VNTR loci

Multiplex PCR mixtures	MIRU locus	Amplicon Length (bp)	Primer sequence (5'-3') (labeling)
A	4	77	GCGCGAGAGCCCGAACTGC (FAM)
			GCGCAGCAGAAACGCCAGC
	26	51	TAGGTCTACCGTCGAAATCTGTGAC
			CATAGGCGACCAGGCGAATAG (HEX)
	40	54	GGGTTGCTGGATGACAACGTGT (NED)
			GGGTGATCTCGGCGAAATCAGATA
B	10	53	GTTCTTGACCAACTGCAGTCGTCC
			GCCACCTTGGTGATCAGCTACCT (FAM)
	16	53	TCGGTGATCGGGTCCAGTCCAAGTA
			CCCGTCGTGCAGCCCTGGTAC (HEX)
	31	53	ACTGATTGGCTTCATACGGCTTTA
			GTGCCGACGTGGTCTTGAT (NED)
C	2	53	TGGACTTGCAGCAATGGACCAACT
			TACTCGGACGCCGGCTCAAAAT (FAM)
	23	53	CTGTCGATGGCCGCAACAAAACG (HEX)
			AGCTCAACGGGTTCGCCCTTTTGTC
	39	53	CGCATCGACAAACTGGAGCCAAAC
			CGGAAACGTCTACGCCCCACACAT (NED)
D	20	77	TCGGAGAGATGCCCTTCGAGTTAG (FAM)
			GGAGACCGCGACCAGGTACTTGTA
	24	54	CGACCAAGATGTGCAGGAATACAT
			GGGCGAGTTGAGCTCACAGAA (HEX)
	27	53	TCGAAAGCCTCTGCGTGCCAGTAA
			GCGATGTGAGCGTGCCACTCAA (NED)

MIRU-VNTR Genescan Analysis. One µl of diluted PCR products was mixed with 10 µl of deionized formamide and 0.2 µl of Genescan ROX-2500 DNA size standard. Denatured samples were subjected to electrophoresis using POP-4 polymer as matrix in a 36cm long capillary array on an ABI PRISM 3100-*Avant* Genetic Analyzer. The key parameters for the capillary electrophoresis were: run temperature = 60 ^{0}C, prerun voltage = 15 kvolts, prerun time = 180 seconds, sample injection voltage = 1 kvolts, sample injection time = 15 seconds, run voltage = 13 kvolts, run time = 2600 seconds. The PCR products were sized by GeneScan™ and Genotyper™ software (Applied Biosystems) using the Custom macro program of Supply *et al*. (2001). The tables used for MIRU-VNTR allele scoring are available at http://www.ibl.fr/mirus/mirus.html. Samples which could not be automatically sized by the software were manually sized and converted to allelic numbers according to the tables.

2.2.4 IS*6110* RFLP typing

The recommended international standard protocol for IS*6110* RFLP (van Embden *et al*., 1993) was followed. Briefly, a 245-bp DNA probe was PCR-amplified using primers INS1 (5'-CGT GAG GGC ATC GAG GTG GCC-3') and INS2 (5'-GCG TAG GCG TCG GTG ACA AA-3') at the 3'-end of IS*6110* insert sequence. The probe was labeled with Enhanced Chemiluminescence (ECL) Direct Nucleic Acid Labeling and Detection System kit (Amersham, Bioscience, Sweden). Genomic DNA was digested with restriction endonuclease *PvuII* (Roche, USA), subjected to electrophoresis separation on a 0.8% agarose gel in Tris-borate-EDTA buffer, and vacuum blotted onto nylon membrane (Amersham Bioscience). Blotted membrane was hybridized overnight at 42^{0}C

in ECL hybridization buffer containing the labeled probe in a hybridization oven (Hybaid, UK). The membrane was then washed and soaked in mixed ECL detection reagents to develop chemiluminescence. The membrane was exposed to a light sensitive ECL Hyperfilm (Amersham Bioscience) for 5-120 minutes (depending on the expected intensity of the reaction) and then developed using Kodak X-ray film developer and fixer. To facilitate the determination of the size of each IS*6110*-hybridizing fragment in computer-assisted analysis for comparison of fingerprints of *M. tuberculosis* strains, a mixture of *HindIII*-digested ϕX174 DNA as an external marker and a mixture of *PvuII*-digested supercoiled DNA ladder and *HaeIII*-digested ϕX174 DNA as an internal marker. The internal marker was added to the wells together with the cleaved mycobacterial DNA and visualized by reprobing the blots with enhanced chemiluminescence kit labeled molecular size marker standards. *PvuII*-digested DNA of *M. tuberculosis* reference strain Mt.14323 was run in parallel as a standard.

The IS*6110* RFLP patterns were analyzed at the National Institute of Public Health and the Environment (RIVM), The Netherlands by using the Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Similarities between RFLP patterns were calculated by using the Dice coefficient and the dendrogram was produced with the unweighted pair group method using arithmetic averages (UPGMA) algorithm.

2.2.5 Calculation of discriminatory power

The Hunter-Gaston Discriminatory Index (HGDI) described by Hunter and Gaston (1988) was used as a numerical index for the discriminatory power of each typing method and strategy. HGDI was calculated using the following formula:

$$HGDI = 1 - \left[\frac{1}{N(N-1)} \sum_{j=1}^{s} n_j (n_j - 1) \right]$$

where N is the total number of strains in the typing scheme, s is the total number of distinct patterns discriminated by each typing method and strategy, and n_j is the number of strains belonging to the jth pattern.

2.2.6 Definition of clustered isolates

Clustered isolates were defined as isolates with identical DNA fingerprint or fingerprints when defined by more than one typing method.

2.3 Results

2.3.1 Genotyping

A total of 364 initial drug-susceptible *M. tuberculosis* isolates from 364 patients were analyzed by spoligotyping, MIRU-VNTR typing, and IS*6110* RFLP typing. All the isolates were successfully typed by spoligotyping and MIRU-VNTR typing. IS*6110* RFLP typing produced 363 readable RFLP patterns.

2.3.2 Genetic diversity by spoligotyping and genotype determination of isolates

Spoligotyping result is shown in Fig. 2.1 (see Appendix 1). The genotypes of the isolates were determined primarily based on their spoligotyping patterns as described before (Filliol et al., 2002; Ferddinand et al., 2004; Kremer et al. 2004). There were 179 isolates had the typical Beijing spoligotype, 17 isolates had atypical Beijing spoligotypes; thus in total there were 196 (53.8%) isolates belonged to Beijing family. Of the remaining 168 non-Beijing isolates, 79 (21.7%) were EAI isolates, 29 (8%) Haarlem isolates, 2 (0.5%)

CAS isolates, 6 (1.6%) LAM isolates, 33 (9.1%) T isolates, and 3 (0.8%) X isolates. Of the above isolates, only 3 EAI isolates (patterns 16, 54 and 55) and 1 Haarlem isolate (pattern 76) (see Fig. 2.1) could not be determined based only on their spoligotypes, their MIRU-VNTR pattern characteristics were refereed (Supply et al., 2001; Sun et al., 2004a). There were 16 (4.4%) isolates did not exhibit any characteristic spoligotypes of the above families. However, the 16 isolates showed clonal characteristics in IS*6110* RFLP and MIRU-VNTR patterns (see Chapter 3 for more details).

As shown in Table 2.2, spoligotyping produced in total 118 distinct patterns; of which, 98 were unique patterns, the remaining 20 were clustered patterns. The largest cluster belonging to the Beijing family contained 179 isolates. The 196 Beijing isolates exhibited only 7 distinct patterns, showing very low discriminatory power (HGDI). But spoligotyping showed high discrimination to non-Beijing isolates. The overall discrimination power (HGDI) of spoligotyping was 0.743.

Table 2.2. Spoligotyping results by *M. tuberculosis* genotypes

Genotypes	No. of isolates	No. of unique patterns	No. of clustered patterns	Clustered isolates	HGDI
Beijing	196	3	4	193	0.165
EAI	79	40	6	39	0.916
CAS	2	2	0	0	1
LAM	6	6	0	0	1
Haarlem	29	18	3	11	0.961
T	33	15	5	18	0.932
X	3	3	0	0	1
S	16	11	2	5	0.967
Overall	364	98	20	266	0.743

2.3.3 Genetic diversity by MIRU-VNTR typing

The 364 isolates showed 176 distinct MIRU-VNTR typing patterns (Table 2.3; see Appendix 2). Of the 176 distinct patterns, 136 were unique patterns, and the remaining 40 were clustered patterns. The largest cluster contained 58 isolates sharing an identical Beijing genotype pattern 223325173533. MIRU-VNTR typing had a better discrimination to non-Beijing isolates than to Beijing isolates, and had the best performance for EAI isolates. A markedly better discrimination for Beijing isolates was achieved by MIRU-VNTR typing than by spoligotyping, although it still clustered 83% (163/196) of Beijing isolates in 23 clusters, with a mean of 7 isolates per cluster. In addition, this typing modality was highly discriminatory to EAI and T families which are known to consist of high number of isolates with low copy numbers of IS*6110*. The overall discrimination power (HGDI) of MIRU-VNTR typing was 0.966 (Table 2.4).

Table 2.4. MIRU-VNTR typing results by *M. tuberculosis* genotypes

Genotypes	No. of isolates	No. of unique patterns	No. of clustered patterns	Clustered isolates	HGDI
Beijing	196	33	23	163	0.894
EAI	79	53	6	26	0.971
CAS	2	2	0	0	1
LAM	6	6	0	0	1
Haarlem	29	12	6	17	0.946
T	33	16	5	17	0.949
X	3	3	0	0	1
S	16	8	3	8	0.942
Overall	364	136	40	228	0.966

2.3.4 Genetic diversity by IS*6110* RFLP typing

As shown in Fig. 2.2, the IS*6110* copy numbers of the *M. tuberculosis* isolates ranged from 0 to 24, a very wide range nearly covered the full range of 0 to 25 copies (van Soolingen, 2001), suggesting the high diversity of *M. tuberculosis* in Singapore. Isolates with high (≥ 6) IS*6110* copies accounted for approximately 85% of the collection. There was less than 1% of isolates had zero copy of IS*6110*, and isolates with single-IS*6110* copy were prevalent, accounting for approximately 9.5% of the collection.

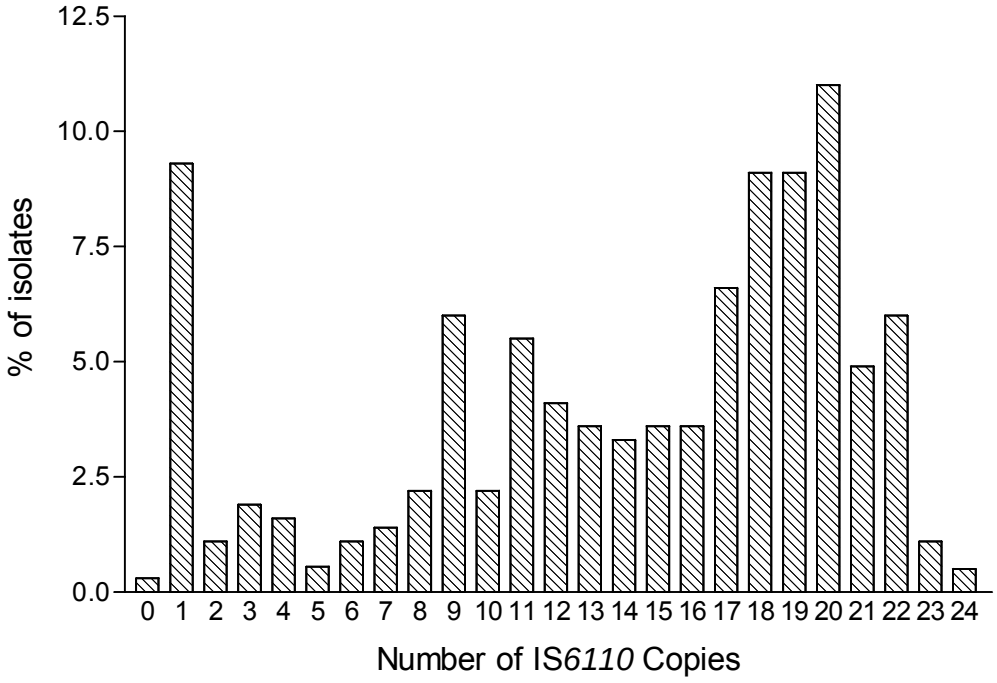

Fig. 2.2. Frequency distribution of *M. tuberculosis* drug-susceptible isolates with different number of IS*6110* copies. IS*6110* copy number ranged from 0 to 24. High (≥ 6) IS*6110*-copy isolates accounted for approximately 85%. Single IS*6110*-copy isolates accounted for approximately 9.5%.

Based on the similarity analysis result of IS*6110* RFLP patterns, IS*6110* RFLP typing generated 288 distinct patterns, 34 cluster patterns and 254 unique patterns. Of the 34 clusters, 30 clusters consisted of in total of 74 high IS*6110*-copy isolates, 3 clusters consisted of in total of 33 single IS*6110*-copy isolates, the remaining 1 cluster consisted of 2 two IS*6110*-copy isolates. The largest cluster contained 19 single IS*6110*-copy isolates. IS*6110* RFLP typing showed the highest discrimination power (HGDI = 0.997) for Beijing isolates although it also defined the most clusters (22 out of 34, 65%) in Beijing isolates. Due to single IS*6110*-copy isolates, IS*6110* RFLP typing was slightly less discriminatory to EAI and T isolates. The overall discrimination power of IS*6110* RFLP typing was 0.995 (Table 2.5).

Table 2.5. IS*6110* RFLP typing results by *M. tuberculosis* genotypes

Genotypes	No. of isolates	No. of unique patterns	No. of clustered patterns	Clustered isolates	HGDI
Beijing	195	137	22	58	0.997
EAI	79	49	6	30	0.942
CAS	2	2	0	0	1
LAM	6	6	0	0	1
Haarlem	29	23	3	6	0.993
T	33	20	2	13	0.894
X	3	3	0	0	1
S	16	14	1	2	0.942
Overall	363	254	34	109	0.995

2.3.5 Multistep typing

The degree of discrimination by combinations of the three methods is listed in Table 2.6. Due to the poor resolution of spoligotyping and of MIRU-VNTR typing to Beijing

strains, the combination of the two methods improved a little in discrimination. The combination of IS*6110* RFLP and spoligotyping had very similar discrimination with the combination of IS*6110* RFLP and MIRU-VNTR; the latter was slightly better. The combination of all three methods did not improve further compared to the combination of IS*6110* and MIRU-VNTR. There were 5 clusters consisted of 14 isolates with high number IS*6110* copies (9 to 22 copies) were further discriminated into 8 unique patterns and 3 clustered patterns by MIRU-VNTR typing, and only one of the 5 clusters was further resolved by spoligotyping.

Table 2.6. Typing results by different combinations of the three methods

Combinations	No. of isolates	No. of unique patterns	No. of clustered patterns	Clustered isolates	HGDI
Spoligotyping & MIRU-VNTR	363	165	34	198	0.970
IS*6110* & spoligotyping	363	280	32	83	0.999
IS*6110* & MIRU-VNTR	363	295	29	68	0.999
All three methods	363	295	29	68	0.999

2.4 Discussion

2.4.1 Genetic diversity and population structure

We have described the genetic diversity and population structure of *M. tuberculosis* in Singapore. Using spoligotyping as the reference method, we found that the seven major worldwide prevalent *M. tuberculosis* families, as defined by decoding an international spoligotyping database (Filliol et al., 2002), were all present in this country. The Beijing genotype was the most prevalent, accounting for 54% of the isolates, followed by

ancestral EAI strains, accounting for 22% of the isolates. The closely related Haarlem and T families accounted for 17%. Isolates from the LAM, the CAS, and the X families were responsible for very small proportion of cases and the CAS was associated with new immigrant from India where the CAS strains are endemic (Bhanu et al., 2002).

In addition to these *M. tuberculosis* families, we have identified a novel clone which showed clonal characteristics in IS*6110* RFLP and MIRU-VNTR patterns but not in spoligotyping patterns, a feature that is different from other genotype strains. This clone has not been systematically described before according to our knowledge, it has therefore been designated as 'S' family and described in Chapter 3.

These proportions of the different *M. tuberculosis* genotypes are based on consecutive samples from 10 residential postal districts in Singapore over a 5-month period in 1994. During this time period there were a total of 519 initial isolates from 519 patients were collected at the Central Tuberculosis Laboratory. Hence the samples used in this study comprise the majority (70%) of samples over a defined time period. Therefore, they are likely to be representative of the whole *M. tuberculosis* population at that time. Although drug-resistant isolates were not included in the study sample, a study based on a collection of all drug-resistant isolates from August 1994 to December 1996 showed that the *M. tuberculosis* genotypes distributed in the similar proportions among drug-resistant isolates (see Chapter 5). Therefore, exclusion of drug-resistant isolates from this study sample is unlikely to have distorted the population structure.

It is not surprising for the predominant prevalence of the Beijing genotype strains because the majority of the Singaporean population is of Chinese race, and that tuberculosis affects the elderly, many of whom are first or second generation immigrants

from mainland China where the Beijing genotype is highly prevalent (van Soolingen et al., 1995; Qian et al., 1999). But in Malaysia and Indonesia, Singapore's closest geographical neighbors, the Beijing genotype represents 24% and 34% of the strains, respectively (Dale et al., 1999; van Crevel et al., 2001). This reflects the association of tuberculosis with population migration.

In addition to China, Beijing strains are also dominant in other Asian countries, such as Mongolia, Vietnam, South Korea, and Thailand (van Soolingen et al., 1995; Anh et al., 2000; Park et al., 2000; Chan et al., 2001; Prodinger et al., 2001), and some areas of Russia (Pfyffer et al., 2001; Drobniewski et al., 2002; Toungoussova et al., 2002, 2003). This phenomenon suggests that the Beijing genotype has selective advantage to spread and/or to establish infection in human over the other genotype strains. van Soolingen et al., (1995) has suggested that it may be related with the massive BCG vaccination in China and the Beijing genotype may represent an 'escape variant' from the protection of BCG vaccination. This has been attracting tremendous research interest to uncover the answer using animal studies (López et al., 2003; Dormans et al., 2004) and molecular epidemiological studies (Anh et al., 2000; van Crevel et al., 2001; Kremer et al., 2005). It is also thought that Beijing strains may be more virulent (Manca et al., 2001; López et al., 2003; Dormans et al., 2004) than other genotype strains due to some intrinsic bacterial factors (Bifani et al., 2002; Manca et al., 2001; Safi et al., 2004; Reed et al., 2004).

2.4.2 Comparison of typing methods

IS*6110* RFLP typing revealed considerable diversity in both the IS*6110* band number pattern and chromosomal position variation. The isolates covered those with zero copy of IS*6110* to those with 24 copies, nearly covering the full range (0-25 copies) of IS*6110* copy number identified worldwide to date (van Soolingen, 2001). Isolates with low IS*6110* copy number (< 6 copies) accounted for 15% of the collection, most of them were single-copy isolates (10%) belonging to either the EAI or T family. This low number of IS*6110* copies, particularly single- or zero-copy, renders IS*6110* RFLP typing inefficient to discriminate strains of these families, as shown by the relatively low discriminatory powers to these two family isolates (Table 2.5). For Beijing genotype isolates, IS*6110* RFLP typing showed the highest discriminatory power because the strains of this family have high number (> 9) of IS*6110* copies, and was superior to MIRU-VNTR typing or spoligotyping.

Among the three typing methods, spoligotyping is the most simple and economic method. It can be easily adapted by either research or clinical laboratories. By decoding a spoligotyping database, major worldwide prevalent strains have been defined with seven evolutionary lineages (Filliol et al., 2002). In this study we have shown its excellent performance in genotype defining of isolate; the vast majority of our isolates could be assigned to each of the *M. tuberculosis* families based on spoligotype alone. This has made it a useful tool for phylogenetic studies (Sola et al., 2001, 2002; Warren et al., 2001). It has been also shown that spoligotyping is useful in molecular epidemiological investigations in the areas where the Beijing genotype is not prevalent (Goguet de la Salmoniere et al., 1997; Sola et al., 1998). Our results showed that it had high discrimination on non-Beijing isolates, therefore support its application to non-Beijing

genotypes. But spoligotyping had very poor resolution to Beijing genotype strains as demonstrated in other studies (van Soolingen et al., 1995; Chan et al., 2001). Due to the high prevalence of Beijing strains in our setting, spoligotyping has limited value to be used as strain-typing method in Singapore.

On population level, MIRU-VNTR typing performed better than spoligotyping, but was not as efficient as IS*6110* RFLP typing. To non-Beijing isolates, MIRU-VNTR typing had a comparable discriminatory power with that of IS*6110* RFLP typing and performed better than IS*6110* RFLP typing for EAI and T family isolates. This agrees with previous studies in developed countries with low tuberculosis incidences and without or with very low proportion of Beijing strains (Mazars et al., 2001; Blackwood et al., 2004) and supports that MIRU-VNTR typing could be an alternative for IS*6110* RFLP typing in these areas (Blackwood et al., 2004). Whereas for Beijing isolates, although MIRU-VNTR typing showed better discrimination than spoligotyping, it still clustered a very significant proportion of Beijing isolates. Thus, MIRU-VNTR typing alone cannot provide sufficient discrimination power in our setting.

The present study represented the first performance of using a genetic analyzer equipped with capillary electrophoresis system for MIRU-VNTR typing analysis. MIRU-VNTR typing based on such a detection system offers high sensitivity, convenience, automation, short turnaround time, and high throughput, and had perfect reproducibility. The digital format of the MIRU-VNTR pattern makes direct inter-laboratory comparisons possible and easy (Sun et al., 2004a).

2.4.3 Strain-typing strategy

As discussed above, no single method could define all unique isolates in our setting; therefore, a multistep typing strategy is needed. We evaluated the combinations of the three typing modalities. The PCR-based combination of MIRU-VNTR typing and spoligotyping clustered more than 50% of the isolates, thus is not applicable in our setting. But this combination may be useful in other areas without or with low Beijing genotype strains (van Deutekom et al., 2005). The combinations of IS*6110* RFLP typing with either MIRU-VNTR typing or spoligotyping showed very similar and the highest discrimination power on the *M. tuberculosis* isolates in our setting. But MIRU-VNTR typing could further subdivide 5 clusters defined by high copy number of IS*6110* RFLP patterns. Although we were unable to demonstrate the relationship of these isolates in the absence of epidemiological data of the patients from whom these isolates were obtained, this case very likely indicates that these isolates are epidemiologically unrelated as demonstrated by a recent Dutch study (van Deutekom et al., 2005). In that study, the authors found that MIRU-VNTR typing can subdivide IS*6110* RFLP and PGRS RFLP defined clusters which consisted of epidemiologically-unrelated patients (van Deutekom et al., 2005). This suggests that MIRU-VNTR typing is more discriminatory than these RFLP typing methods in this case and clusters defined by both IS*6110* RFLP and MIRU-VNTR typing can more accurately reflect transmission event. These findings emphasize the potential use of MIRU-VNTR typing to refine IS*6110* RFLP typing in order to better understand transmission routes and support contact tracing. Furthermore, MIRU-VNTR typing had a better performance in subdividing single-IS*6110* isolate clusters than spoligotyping.

In view of the advantages and the excellent discrimination power of the combination of IS*6110* RFLP typing and MIRU-VNTR typing, this combination could be a suitable modality for *M. tuberculosis* strain-typing in Singapore using MIRU-VNTR typing as a first-line screening method.

CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF A NOVEL *M. TUBERCULOSIS* CLONE BY MULTIPLE GENETIC MARKERS

3.1 Introduction

In the past 15 years, a considerable amount of information has been obtained on the genetic diversities and population structures of *M. tuberculosis* worldwide. Analyses by various genetic markers indicate that *M. tuberculosis* evolves and disseminates by clonal expansion (Warren et al., 2001; Supply et al., 2003; Baker et al., 2004) which results in great geographic variations in the distribution of *M. tuberculosis* evolutionary lineages (Sola et al., 2002; Filliol et al., 2002; Baker et al., 2004).

Thus far, the most optimal phylogenetic marker for defining the evolutionary clonalities of *M. tuberculosis* is the direct repeat (DR) locus of the bacillary genome which is conserved only in the subspecies of *M. tuberculosis* complex (Hermans et al., 1991). Based on spoligotype, a type of DNA fingerprint of the DR region generated by spoligotyping (Kamerbeek et al., 1997), the global *M. tuberculosis* isolates can be well assigned into seven major evolutionary lineages and some minor ones, each family is defined by common family characteristics of spoligotype (Filliol et al., 2002; Sebban et al., 2002, Kremer et al., 2004). Studies based on grouping of *M. tuberculosis* isolates by spoligotype has revealed useful information for understanding of the evolutionary history, the phylogeographical distribution, the global transmission of the bacilli (Sola et al., 1999, 2001; Warren et al., 2002a; Dale et al., 2003; Filliol et al., 2003, Baker et al., 2004), and family-specific disease phenotypes and pathogenesis (Glynn et al., 2002; Bifani et al., 2002, Baker et al., 2004).

However, in our study samples of drug-susceptible (Chapter 2) and drug-resistant *M. tuberculosis* (Chapter 5) collected from Singapore, there were 27 isolates (16 susceptible and 11 resistant isolates) which showed clonal characteristics in IS*6110*

RFLP and MIRU-VNTR patterns, but not in spoligotyping patterns. These isolates represent a novel *M. tuberculosis* family clone, and therefore have been designated as the "S" family of *M. tuberculosis*.

3.2 Materials and Methods

3.2.1 Mycobacterial isolates

Twenty-seven clinical *M. tuberculosis* isolates (16 drug-susceptible isolates and 11 drug-resistant isolates) were selected from the collections of drug-susceptible and drug-resistant *M. tuberculosis* as described in Chapters 2 and 5.

3.2.2 DNA fingerprinting

IS*6110* RFLP typing, spoligotyping, and MIRU-VNTR typing was performed as described in Chapter 2.

3.2.3 Genomic insertion and deletion analysis

The isolates were analyzed for the insertion of IS*6110* copy at the A1 position of the *dnaA-dnaN* intergenic region of *M. tuberculosis* genome, and the region of difference 9 (RD9) and flanking the *M. tuberculosis* specific deletion 1 (TbD1) region as described before (IS*6110* insertion in *dnaA-dnaN* region analysis was described by Kurepina et al., 1998 and Milan et al., 2004; RD9 and TbD1 analysis was described by Brosch et al., 2002).

3.2.4 *kat*G^{463} and *gyr*A^{95} single nucleotide polymorphism (SNP) analysis

The *kat*G^{463} and *gyr*A^{95} single nucleotide polymorphism (SNP) analysis has been previously described (Sreevatsan et al., 1997). After PCR amplification, each PCR product was subjected to cycle DNA sequencing using the sequencing primer and ABI PRISM® BigDye™ V3.0 Cycle Sequencing kit (Applied Biosystems, Foster City). The cycle sequencing products were purified using Dye-terminator removal kit (Qiagen) and ethanol precipitation before being analyzed using ABI PRISM® 3100-*Avant* Genetic Analyzer. DNA sequencing was performed using ABI PRISM 3100-*Avant* Genetic Analyzer (Applied Biosystems).

3.2.5 Phylogenetic analysis

PAUP (Phylogenetic Analysis Using Parsimony and other methods) software 4.0 beta version (Sinauer Associates, Inc., Sunderland, Mass) was used to perform phylogenetic analysis using the UPGMA (unweighted pair group method using arithmetic averages) algorithm based on MIRU-VNTR typing data. TreeView (version for Windows, available at http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) was used to print out the dendrogram generated by PAUP analysis.

3.2.6 Allelic diversity

The MIRU-VNTR locus allelic diversity (h) was calculated as $h = 1-\sum x_i^2$, where x_i is the frequency of the ith allele at the locus (Graur and Li. 2000).

3.3 Results

3.3.1 Genotypic analysis

IS*6110* RFLP fingerprints showed that 25 of the 27 SG clone isolates carried 13 to 17 IS*6110* copies. Of the remaining 2 drug-resistant isolates, one (I08) had 20 bands and the other (ISE1) had 21 bands. A number of bands were shared by all or by most of the isolates (Fig. 3.1). The patterns were highly homogenous. IS*6110* RFLP similarity analysis showed that these isolates shared greater than 70% of similarity. In spite of this, IS*6110* RFLP typing still generated 25 distinct patterns.

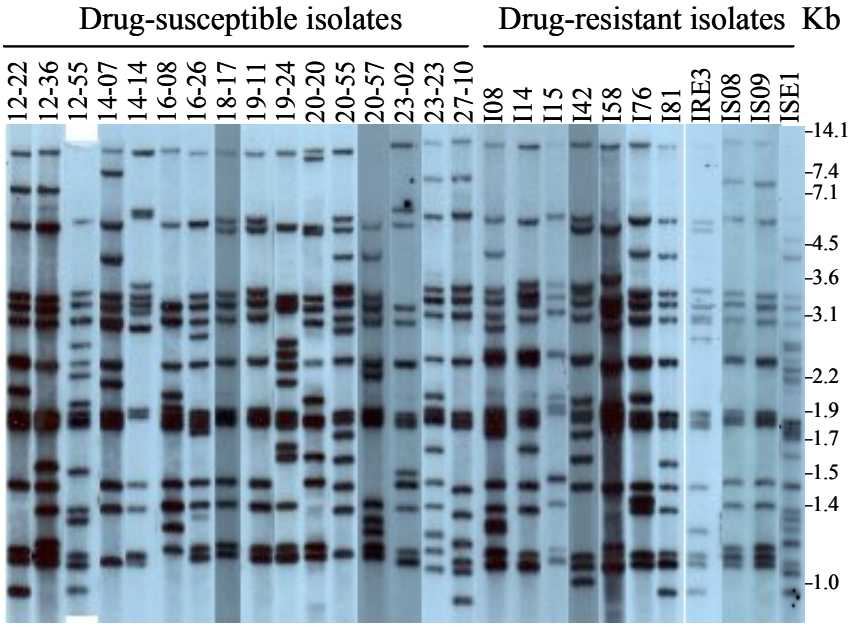

Fig. 3.1. IS*6110* RFLP patterns of the S clone isolates. Twenty-six of the 27 isolates the S clone contained 13 to 17 IS*6110* copies, and multiple IS*6110* bands are shared by all or most of the isolates. Sizes of standard DNA fragments are indicated to the right in kilobase pair.

Similarly, as shown in Table 3.1, the MIRU-VNTR patterns were also highly homogenous and restricted. The 27 isolates exhibited 14 distinct MIRU-VNTR patterns. In 6 of the 12 MIRU-VNTR loci (loci 2, 4, 20, 23, 24, 27), the allelic numbers were

invariable. The most variable MIRU-VNTR locus was locus 10 (allelic diversity = 0.74), which alone could define 7 of the 14 distinct patterns. Moreover, the locus 10 was characterized by a high repetitive allele number; all but one of the isolates had ≥ 5 allelic copies.

Table 3.1. MIRU-VNTR patterns of the S clone isolates

Isolates	2	4	10	16	20	23	24	26	27	31	39	40
12-22	2	2	7	2	2	5	1	7	3	4	3	3
16-26	2	2	7	2	2	5	1	7	3	4	3	3
23-02	2	2	7	2	2	5	1	7	3	4	3	3
I08	2	2	7	2	2	5	1	7	3	4	3	3
I14	2	2	7	2	2	5	1	7	3	4	3	3
I42	2	2	7	2	2	5	1	7	3	4	3	3
I76	2	2	7	2	2	5	1	7	3	4	3	3
I81	2	2	7	2	2	5	1	7	3	4	3	3
IRE3	2	2	7	2	2	5	1	7	3	4	3	3
12-36	2	2	7	2	2	5	1	7	3	4	3	4
18-17	2	2	5	2	2	5	1	7	3	4	3	3
19-11	2	2	5	2	2	5	1	7	3	4	3	3
19-24	2	2	5	2	2	5	1	7	3	4	3	3
16-08	2	2	6	2	2	5	1	7	3	4	3	3
I15	2	2	6	2	2	5	1	7	3	4	3	3
23-23	2	2	3	2	2	5	1	7	3	4	3	3
27-10	2	2	9	2	2	5	1	7	3	4	3	3
IS08	2	2	10	3	2	5	1	7	3	4	3	3
IS09	2	2	10	3	2	5	1	7	3	4	3	3
ISE1	2	2	7	3	2	5	1	7	3	4	3	3
12-55	2	2	8	2	2	5	1	7	3	4	3	2
14-14	2	2	8	2	2	5	1	7	3	4	3	3
20-20	2	2	8	2	2	5	1	7	3	4	4	3
20-55	2	2	8	2	2	5	1	7	3	4	4	3
20-57	2	2	8	2	2	5	1	7	3	4	2	2
14-07	2	2	8	2	2	5	1	6	3	4	3	3
I58	2	2	8	2	2	5	1	6	3	3	3	3

As shown in Fig. 3.2, spoligotyping generated a total of 15 distinct spoligotypes, 3 clustered patterns (P1, P2, and P6) and 12 unique patterns (P3-P4, P7-P15). The pattern P1 which had all the 43 spacers conserved was previously identified as a spoligotype of

an *M. africanum* strain (strain number 960762) in the study by Viana-Niero et al. (2001). The pattern P6 was also previously identified from *M. tuberculosis* strains in Houston, the United States (Soini et al., 2000). Despite the high homogeneity of the S clone isolates in both IS*6110* RFLP and MIRU-VNTR patterns, no common family characteristics could be drawn from the spoligotypes of these isolates (Fig. 3.2). Therefore in this regard, the S clone is different from the other *M. tuberculosis* families characterized in previous studies (Filliol et al, 2002; Sebban et al., 2002; Douglas et al., 2003; Sun et al., 2004b).

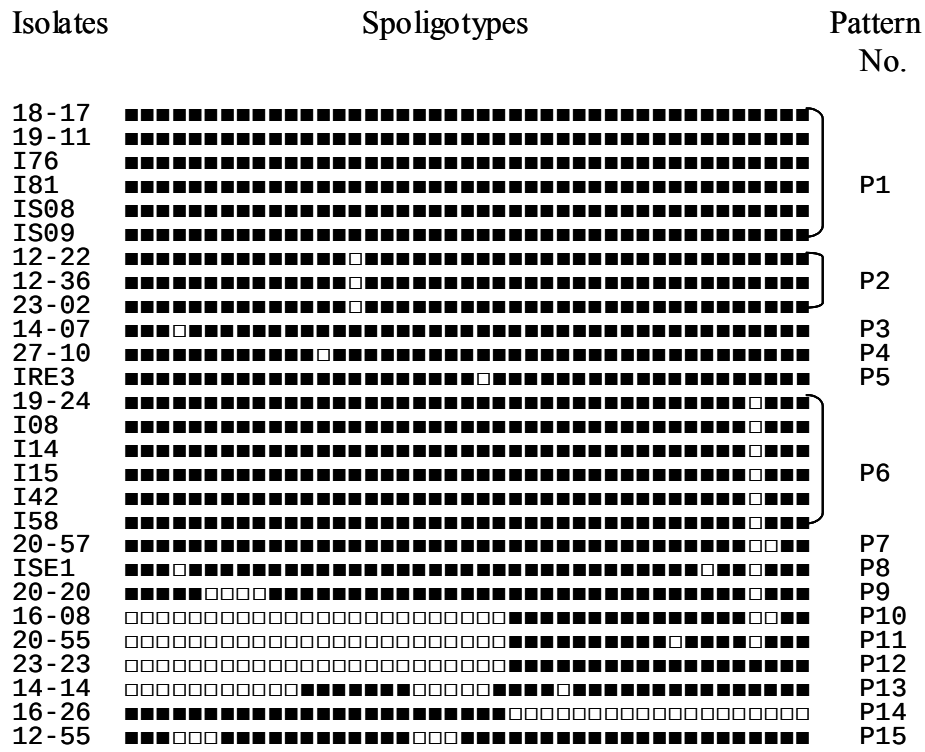

Fig. 3.2. Spoligotypes of the S clone isolates. The 43 spacer formats of spoligotypes of the 27 S clone isolates are shown. ■ denotes the presence of the spacer and □ indicates the absences of the spacer. Shared spoligotype patterns (P1, P2, and P6) were seen within several subgroups of isolates, but no common spoligotype characteristics could be drawn for all the isolates.

In other genetic markers, at the A1 position of the *dnaA-dnaN* region of *M. tuberculosis* genome, an IS*6110* insert was detected in all the 27 isolates, and all the isolates had RD9 conserved and TbD1 deleted. In regard to the SNP of *kat*G^{463} and *gyr*A^{95}, all the isolates showed *kat*G^{463} CTG (Leu) and *gyr*A^{95} ACC (Thr) polymorphisms, corresponding to the principal genetic group 1 (Sreevatsan et al., 1997).

3.3.2 Phylogenetic analysis

To explore the phylogenetic position of the S clone in the *M. tuberculosis* complex, we built a genetic tree (Fig. 3.3) based on pooled MIRU-VNTR typing data of the S clone isolates (Table 3.1) and that of a global collection of *M. tuberculosis* complex strains (Kremer et al., 1999) which have been typed in a previous study (Supply et al., 2001). On the dendrogram, all the S family isolates fell into a clade belonging to one of the modern *M. tuberculosis* branches. The S clone, the Beijing family, and the clades of the CAS family are diverged from the same branch which represents the modern *M. tuberculosis* of principal genetic group 1 of the *M. tuberculosis* complex (Sreevatsan et al., 1997). Interestingly, another *M. africanum* strain in the global collection of *M. tuberculosis* complex strains (strain 100) fell into the clade of the S clone and shared an identical MIRU-VNTR pattern (226225173433) with the S clone strains 16-08 and I15.

Fig. 3.3. Phylogenetic position of the S clone. This dendrogram was generated using the UPGMA algorithm based on pooled MIRU-VNTR typing data of this study (Table 3.1) with that of a global collection of *M. tuberculosis* complex strains which were previously MIRU-VNTR typed (Supply et al., 2001). *M. canettii* (strain 116) was used as an outgroup. Linkage distance is indicated at the left bottom corner.

3.4 Discussion

The discovery of the S genogroup has added information to the genetic diversity of *M. tuberculosis*. Due to the small proportion of the S isolates and limited demographic and epidemiological data that were available, we did not find any clinical or epidemiological significance of these isolates. The proportions of the S isolates in drug-sensitive and -resistant *M. tuberculosis* were the same, suggesting that they were not more related with drug-resistance.

However, the S genogroup provides an interesting evolutionary model for investigating the evolution of the DR region of *M. tuberculosis* genome. Spoligotyping is based on the polymorphism of the DR region and currently serves as a reference method for defining evolutionary lineages of *M. tuberculosis* because family characteristics of spoligotype are well conserved among strains of each family (Filliol et al., 2002; Sebban et al., 2002, Douglas et al., 2003; Kremer et al., 2004). However, although a number of clonal characteristics in IS*6110* RFLP and MIRU-VNTR patterns suggest that the 27 isolates were derived from a common progenitor, these isolates did not show any family characteristics in their spoligotypes that can be used to group these isolates as a family like the other known *M. tuberculosis* families. This represents an exception from a common evolutionary mechanism. In addition, it has been suggested that the polymorphism of the DR region results from the irreversible deletion of direct variable repeat (DVR) (van Embden et al., 2000; Warren et al., 2002a; Sampson et al., 2003) and the deletion may be mediated by IS*6110* insertion (Warren et al., 2002a; Sampson et al., 2003). Most of the S isolates had the 43 DVRs conserved or only lost 1 to 3 DVRs, therefore, these isolates are valuable for investigating this potential evolutionary

mechanism. Thus, further studies on the structure of the DR locus of these isolates would shed more light into the evolutionary mechanism of the DR region.

The S family isolates were genetically close to the Beijing genotype as demonstrated by genetic relatedness in their IS*6110* RFLP and MIRU-VNTR patterns with that of Beijing strains, and by the presence of a IS*6110* insert in the *dnaA-dnaN* region of the S family isolates because all Beijing strains have a IS*6110* copy inserted in this region (Kremer et al., 2004). However, the spoligotypes of the S isolates indicate that these isolates did not belong to the Beijing family. Furthermore, the high allelic diversity and the high repetitive allele number in MIRU-VNTR locus 10 were very different features from those of Beijing strains.

Phylogenetic analysis suggests that the S family might share a common progenitor with the Beijing and CAS families as they are diverged from the same branch of the MIRU-VNTR genetic tree (Fig. 3.3). In phylogenetic markers of TbD1 and the SNP of *kat*G^{463} and *gyr*A^{95}, the S family isolates exhibited the same evolutionary traits as Beijing and CAS strains did. TbD1 is deleted from the genomes of modern evolutionary lineages of *M. tuberculosis*, including the known families of Beijing, CAS, LAM, Haarlem, T, and X, but conserved in the ancestral EAI family strains of *M. tuberculosis* and the other taxons of the *M. tuberculosis* complex (Brosch et al., 2002), thus a specific marker to differentiate ancestral and modern *M. tuberculosis*. Based on the SNP of *kat*G^{463} and *gyr*A^{95}, the *M. tuberculosis* complex isolates are divided into three principal genetic groups (Sreevatsan et al., 1997). The *M. tuberculosis* families of Beijing, CAS, and EAI belong to the principal genetic group 1. Taken together, all the results suggest that the S clone is a member of the modern *M. tuberculosis* of the principal genetic group

1 (Sreevatsan et al., 1997), and the Beijing family is the closest evolutionary lineage to the S family.

Interestingly, two previously characterized *M. africanum* strains, the strain 960762 in the study by Viana-Niero et al. (2001) and the strain 100 in the Kremer's collection (Kremer et al., 1999; Supply et al., 2001), were found to share either an identical spoligotype or an identical MIRU-VNTR pattern with isolates of the S clone. The strain 100 in the Kremer's collection was originally identified as a representative of *M. africanum*, designated so was based solely on its biological phenotype (Kremer et al. 1999). However, this strain was genetically distant from another *M. africanum* strain (strain 92) in the same collection based on their IS*6110* RFLP patterns (Kremer et al., 1999) and MIRU-VNTR patterns (Supply et al., 2001, and Fig. 3.3 in this study), and the allelic number of MIRU-VNTR locus 24 of the strain 92 indicates that it belonged to an ancestral taxon of the *M. tuberculosis* complex, whereas that of the strain 100 indicates that it belonged to a modern evolutionary lineages of *M. tuberculosis* (Supply et al., 2001; Sun et al., 2004a). Findings from another study (Brosch et al., 2002) also suggest that the strain 100 was a *M. tuberculosis* strain instead of a *M. africanum* strain because the strain 100 was different from a group of *M. africanum* strains in two critical genomic deletion markers, RD9 and TbD1. RD9 is absent from *M. africanum, M. bovis,* and *M. microti*, but present in *M. tuberculosis* and *M. canettii*. TbD1 is present in *M. africanum, M. bovis, M. microti* and *M. canettii*, as well as the ancestral *M. tuberculosis* EAI strains, but absent from the modern family strains of *M. tuberculosis* (Brosch et al., 2002). The strain 100 was RD9 positive but TbD1 negative. These molecular evidence unanimously indicate that the strain 100 is a member of modern *M. tuberculosis*. Accordingly, some

members of the *M. tuberculosis* S family may phenotypically present as *M. africanum*, an interesting phenomenon that deserves further investigation.

CHAPTER 4

ASSOCIATION OF *M. TUBERCULOSIS* BEIJING GENOTYPE WITH TUBERCULOSIS RELAPSE

4.1 Introduction

The *M. tuberculosis* Beijing genotype family constitutes a group of genetically homogeneous strains, presumably due to recent clonal expansion (van Soolingen et al., 1995). Beijing genotype strains are globally widespread with dominant prevalence in a number of East Asian countries, and associated with drug-resistance in Vietnam, New York, Cuba, and Estonia (van Soolingen et al., 1995; Bifani et al., 2002; Glynn et al., 2002). Moreover, in Vietnam the Beijing genotype was found to be associated with younger patients, and based on this, it has been proposed that the Beijing genotype is an emerging pathogen in this country (Anh et al., 2000). But the relationship of the Beijing genotype with patient's age remains a matter of debate because the association has not been found in other areas (Glynn et al., 2002). Also in Vietnam, the Beijing genotype was recently reported to be associated with treatment failure and tuberculosis relapse (Lan et al., 2003). All these findings are important for understanding the Beijing genotype, it is therefore necessary to confirm the findings in different settings because epidemiological studies may be biased by many potential confounding factors.

We have described the dominant prevalence of Beijing strains in Singapore in Chapter 2. In this retrospective study, we aimed to demonstrate the relationships of Beijing strains with tuberculosis relapse and with patient's age in our setting.

4.2 Materials and Methods

4.2.1 Study subjects and mycobacterial isolates

Study subjects were the 364 patients who had drug-susceptible tuberculosis and their isolates have been analyzed in Chapter 2. Demographic data of age, sex, and ethnicity as

well as the information of current episode (recurrence or first episode) were obtained retrospectively from the National Tuberculosis Registry database. Mycobacterial isolates were the same as described in Chapter 2 and the genotyping results were used in this chapter.

4.2.2 Definitions for recurrent, relapsed, and reinfected tuberculosis

Patients who had tuberculosis and were completely bacteriologically negative with anti-tuberculosis treatment 2 years prior to the current episode were categorized as recurrent cases. Recurrent cases who were infected with clustered *M. tuberculosis* isolates by IS*6110* and MIRU-VNTR typing were defined as exogenously re-infected patients, the remaining recurrent cases who were infected with a unique strain determined by IS*6110* and MIRU-VNTR typing were defined as relapsed cases. The non-recurrent and re-infected cases were categorized as non-relapsed cases.

4.2.3 Statistical analysis

χ^2 test was used to analyze categorical variables, and Spearman's correlation test was used to analyze trend correlation of categorical variables. Odds ratio (OR) with 95% confidence interval (CI) was calculated. Student's *t* test was used to compare normally distributed continuous variables. Multivariate logistic regression was used to adjust for potential confounding factors. A *p* value of <0.05 was considered statistically significant.

4.3 Results

Of the 364 patients, 196 (53.8%) were infected with Beijing genotype isolates. Information on patient's age, sex, ethnicity, and current episode were available for 335 patients. The 29 patients with missing data had a similar proportion of Beijing genotype strains to the others, and the dates of specimen acquisition were evenly distributed throughout the sampling period. Therefore, the exclusion of the 29 patients from the total sample is unlikely to introduce bias into the study.

The 335 patients (243 males, 72.5%) ranged in age from 6 to 91 years, with a mean age of 50 years (SD = 19). Fifty-two of the 335 patients had recurrent tuberculosis, the remaining 283 had first episode disease. Of the recurrent cases, 7 (5 were infected with Beijing strains, 2 with non-Beijing strains) were regarded as having exogenous re-infection as they were members of seven IS*6110* and MIRU-VNTR fingerprint defined clusters, the remaining 45 recurrent patients (37 males, 82.2 %) were categorized as relapsed cases. Beijing strains were found in 32 (71.0%) of the 45 relapsed cases and in 148 (51.0%) of the 290 non-relapsed (283 first episode and 7 re-infected) cases. As shown in Table 4.1, univariate analysis revealed that there was an association between Beijing strains and tuberculosis relapse (OR, 2.36; 95% CI, 1.20-4.64; $p = 0.019$). The mean age was 61 years (SD = 16) in the relapsed group, but it was 48 years (SD = 19) in the non-relapsed group, the difference is statistically significant (t test, $p = 0.0001$). All but one relapsed case were older than 30 years, and about a half of relapsed cases were ≥ 65 years. Tuberculosis relapse was found to be strongly associated with older patients by univariate analysis ($p < 0.001$). However, no significant association of tuberculosis relapse with sex ($p = 0.166$) and ethnicity ($p = 0.375$) was observed.

Multivariate logistic regression analysis, which included genotypes (dichotomous as Beijing and non-Beijing) and age (continuous variable), confirmed the significant association of Beijing genotype with tuberculosis relapse (OR, 2.64; 95% CI, 1.30-5.34; $p = 0.005$). Patient's age was also independently associated with tuberculosis relapse, the odds that an older patient had relapsed tuberculosis increased 4% over that of a younger patient with each year of age (OR, 1.04; 95% CI, 1.02-1.06; $p < 0.001$).

The relationship between *M. tuberculosis* Beijing genotype and patient's age was also examined. The mean ages were 49 (SD = 20) and 51 (SD = 18) for the patients infected with Beijing and non-Beijing strains respectively. There was no association between the Beijing genotype and patient's age in our study ($p = 0.244$).

4.4 Discussion

Recurrent tuberculosis may develop as the result of reactivation of the endogenous primary infection (relapse) or as a result of a recent exogenous infection (re-infection) (van Rie et al., 1999). The conclusive method to differentiate these two events from each other is to fingerprint *M. tuberculosis* isolates of the primary and recurrent episodes. If the paired isolates of primary and recurrent episodes of one patient are identical (or very similar) in their DNA fingerprints, the recurrent event is regarded as a reactivation; otherwise, if the paired isolates exhibit different DNA fingerprints, the recurrent event is considered to be re-infection. As strain-typing was not done in the previous episodes of tuberculosis for the recurrent cases in this study, we were unable to define these cases as relapse or re-infection by this method. Nevertheless, re-infection is an event of recent transmission from person to person; isolates involved in this event are epidemiologically

Table 4.1. Analysis of relapsed and non-relapsed tuberculosis cases based on the *M. tuberculosis* genotypes and demographic factors

Genotypes	No. of patients	Patient (%)		Univariate analysis[a]		Multivariate analysis[b]	
		Relapsed	Non-relapsed	OR (95% CI)	*p*	OR (95% CI)	*p*
Genotype					0.019		0.005
Beijing	180	32 (17.8)	148 (82.2)	2.36 (1.20-4.64)		2.64 (1.30-5.34)	
Non-Beijing	155	13 (8.4)	142 (91.6)	1		1	
Age group (y)					0.000		
< 25	35	1 (2.9)	34 (97.1)	1			
25-44	110	6 (5.5)	104 (94.5)	1.96 (0.30-12.7)			
45-64	103	17 (16.5)	86 (83.5)	6.72 (1.09-40.8)			
> 64	87	21 (24.1)	66 (75.9)	10.8 (1.76-65.3)			
Age (y)[c]	335	45 (13.4)	290 (86.6)			1.04 (1.02-1.06)	0.000
Sex					0.166		
Male	243	37 (15.2)	206 (84.4)	1.89 (0.86-4.14)			
Female	92	8 (8.7)	84 (91.3)	1			
Ethnicity					0.375		
Chinese	248	32 (12.9)	216 (87.1)	1			
Malay	54	8 (14.8)	46 (85.2)	1.18 (0.52-2.67)			
Indian	21	5 (23.8)	16 (76.2)	2.11 (0.75-5.96)			
Unknown	12	0 (0)	12 (100)	-			

[a] χ^2 and Spearman's correlation. OR, odds ratio; CI, confidence interval.
[b] Logistic regression analysis. OR for genotype and age is adjusted by each other.
[c] Used as continuous variable in Logistic regression analysis. OR (1.04) indicates that the odds of relapse increases 4% every one year older.

related, thus likely showing identical or nearly identical DNA fingerprints. Isolates from reactivated cases are likely to be distinct from each other. Based on this notion, we have defined each of the recurrent cases as relapse or re-infection and found that *M. tuberculosis* Beijing genotype was associated with tuberculosis relapse in Singapore. This observation agrees with the finding of the Vietnamese study (Lan et al., 2003).

The limitation of our definitions for relapse and non-relapse is the risk of misclassification of true relapsed cases into re-infection or true re-infected cases into relapse. However this possibility seems unlikely to counter the association between the Beijing genotype and tuberculosis relapse for several reasons. Firstly, the relative contribution of exogenous re-infection to recurrent tuberculosis depends on the incidence of tuberculosis in a community (van Rie et al., 1999; Jasmer et al., 2004). Although the incidence in Singapore was moderate (49 per 100,000 people) in 1994, re-infection appeared to be not significant because in 21 drug-resistant tuberculosis patients who had multiple isolates (the time intervals between the first and last isolates for a given patient ranged from 45 to 815 days, with a mean of 258 days) sampled during the period of August 1994 to December 1996, all subsequent isolates had identical or nearly identical (only one band difference) IS*6110* RFLP patterns and identical MIRU-VNTR patterns with the respective initial isolate (data not shown), indicating that none of these patients had exogenous reinfection. Secondly, the findings that the mean age of the relapsed group was significantly older than that of the non-relapsed group and relapse was strongly correlated with older patients suggest that the recurrences in the relapsed group were more likely due to endogenous reactivation rather than exogenous reinfection. Thirdly, by our definition of re-infection, more recurrent cases infected with Beijing

strains (5 cases) were classified as re-infected cases compared to those infected with non-Beijing strains (2 cases).

We did not find an association between Beijing strains and patient's age in this study. This observation is consistent with previous ones obtained from Hong Kong, Thailand, Indonesia, and Estonia (Glynn et al., 2002), but disagrees with the previous study performed in Vietnam (Anh et al., 2000). The disparity between the studies was possibly due to variations in overall population structure in these settings; for example, the < 30-year population accounted for approximately 47% of the total population in Singapore in 1994, the same age group of population accounted for about 62% of the total population in Vietnam in 2000 (U.S. Census Bureau, 2005) http://www.census.gov/ipc/www/idbpyr.html). Thus, the association between Beijing strains and younger patients in Vietnam might be because it had higher proportion of younger population. As such, when comparing such data from different settings, study samples should be standardized with respective national population data.

CHAPTER 5

MOLECULAR EPIDEMIOLOGY OF DRUG-RESISTANT TUBERCULOSIS: TRANSMISSION ANALYSIS AND ASSOCIATIONS BETWEEN DRUG-RESISTANT PHENOTYPES AND GENOTYPES OF *M. TUBERCULOSIS*

5.1 Introduction

Drug-resistant tuberculosis is a major threat to global control of the epidemic. According to a recent WHO report (WHO, 2004), drug-resistant tuberculosis is highly prevalent and emerging in multiple areas of the world. Drug-resistant tuberculosis can be disseminated with population immigration from areas with high incidence to areas with low incidence (Granich et al., 2005).

"Resistance among new cases" results from the transmission of drug-resistant *M. tuberculosis* isolate from a patient to another person. The incidence of "resistance among new cases" in a community is an indicator of transmission within the community. "Resistance among previously treated cases" results from man-made factors, such as improper medical management, inappropriate prescription, or poor adherence of patients to treatment, *etc*.. The level of "resistance among previously treated cases" reflects the performance of on-going tuberculosis control program (WHO, 1997, 2000b).

Drug-resistant tuberculosis has been a persistent problem in Singapore with an incidence fluctuating in a range of 4.2% to 6.7% from 1994 to 2004 (Boudville et al., 1997; Communicable Disease Surveillance Report 2004, the Department of Clinical Epidemiology, Tan Tock Seng Hospital). However, little is known about the transmission dynamics of drug-resistant tuberculosis in this large urban center. In this population-based study, we estimated the relative contribution of recent transmission to drug-resistant tuberculosis on the basis of molecular genotyping analysis, and examined the relationships between drug-resistant phenotypes and genotypes of *M. tuberculosis.*

5.2 Materials and Methods

5.2.1 Mycobacterial DNA samples

A DNA sample bank of 271 drug-resistant *M. tuberculosis* isolates was used for *M. tuberculosis* genotyping. These isolates were consecutively collected between August 1994 and December 1996 from the Central Tuberculosis Laboratory, Singapore General Hospital, Singapore. The collection of isolates represented the population of drug-resistant *M. tuberculosis* in Singapore during the study period. Drug-susceptibility testing was performed using the BACTEC 460 radiometric method (Becton Dickinson, Towson, Md). The concentrations tested were 0.1 μg/ml for isoniazid, 2 μg/ml for rifampin, 6 μg/ml for streptomycin, and 7.5 μg/ml for ethambutol. The drug-susceptible isolates described in Chapter 2 were used as a control sample.

5.2.2 Genotyping analysis

The genotyping methods of spoligotyping, MIRU-VNTR, and IS*6110* RFLP, as well as the similarity analysis of IS*6110* RFLP patterns are the same as described in Chapter 2. The genotype of the isolates was determined as described in Chapter 2. The definition of transmission cluster is the same as described in Chapter 2 and transmission clusters were defined using both IS*6110* RFLP and MIRU-VNTR patterns.

5.2.3 Statistical analysis

Differences between groups were tested by χ^2 test (or Fisher's exact test) and multivariate logistic regression. Odds ratio (OR) with 95% confidence interval (CI) was calculated. A p value of < 0.05 was considered statistically significant.

5.3 Results

5.3.1 Frequencies of isolates by drug-resistant patterns

A total of 271 drug-resistant isolates (244 initial isolates and 27 subsequent isolates) were collected, which were from 244 patients. Among the 244 initial isolates, 10 isolates (8 monoresistance to INH, 1 monoresistance to STR, 1 resistance to both INH and STR) were excluded because DNA samples were not available. Table 5.1 shows the drug-resistant patterns of the remaining 234 initial isolates and the frequencies of isolates of each resistant pattern. A total of 168 (71.8%) isolates were resistant to at least INH, 103 isolates (44.0%) were resistant to at least STR, 48 (20.5%) isolates were resistant to at least RIF, and 16 (6.8%) isolates were resistant to at least EMB. Forty-one (17.5%) isolates were MDR.

Table 5.1. Drug-resistant patterns of 234 *M. tuberculosis* isolates[a]

Resistance to:	No. of isolates (%)
INH	93 (39.7)
RIF	7 (3.0)
STR	57 (24.4)
EMB	2 (0.9)
INH, RIF	21 (9.0)
INH, RIF, EMB	5 (2.1)
INH, RIF, STR	10 (4.3)
INH, RIF, STR, EMB	5 (2.1)
INH, STR	30 (12.8)
INH, EMB	3 (1.3)
INH, STR, EMB	1 (0.4)

Note. [a]Data shown are absolute numbers with percentage of the group total in brackets.
INH, isoniazid; RIF, rifampin; STR, streptomycin; EMB, ethambutal

5.3.2 Genotype determination of isolates

Among the 234 initial isolates, 216 had typing results for all the three typing modalities. Of the remaining 18 isolates, 9 had only IS*6110* RFLP typing results (one isolate carried single IS*6110* copy, 8 isolates carried ≥ 9 IS*6110* copies), the remainder had both spoligotyping and MIRU-VNTR typing results. Each of the 224 isolates which had spoligotyping and MIRU-VNTR typing results was assigned to one of the eight *M. tuberculosis* genotypes as described in Chapters 2 and 3. The genotypes of the 8 isolates that had only IS*6110* RFLP typing results were determined based on their IS*6110* RFLP patterns by similarity comparison with the isolates with known genotypes. There were 142 (60.1%) isolates belonged to the Beijing genotype, 45 (19.2%) isolates belonged to the EAI genotype. In the remaining 48 isolates, 2 (0.9%) belonged to the CAS family, 11 (4.7%) belonged to the S family, 13 (5.6%) belonged to the Haarlem family, 3 (1.3%) belonged to the LAM family, and 17 (7.3%) belonged to the T family. No X genotype isolate was found from this drug-resistant sample.

5.3.3 Transmission analysis of drug-resistant tuberculosis

Of the 234 isolates, 3 Beijing isolates which had shared MIRU-VNTR patterns but did not have IS*6110* RFLP typing result and 1 EAI isolate which had shared single IS*6110* band pattern but did not have MIRU-VNTR and spoligotyping results were excluded from clustering analysis because the status of these isolates, i.e., whether they were unique or clustered, could not be determined. In the remaining 230 isolates, 180 were unique isolates, 50 were clustered isolates in 21 clusters.

The characteristics of the clustered isolates are listed in Table 5.2. Sixteen (76%) of the 21 clusters were comprised of Beijing isolates. The largest cluster (cluster 12) contained 6 isolates. Drug-resistant patterns of isolates within 15 clusters (1 through 11 and 18 through 21) were identical. Drug-resistant patterns of isolates within 3 clusters (clusters 13, 16, and 17) were entirely different, and those of isolates in clusters 14 and 15 differed for one drug. Cluster 12 can be subdivided into two clusters based on the resistant patterns of the isolates, isolates 12A and 12B as one cluster, the remaining 12C through 12F as the other.

By assuming that there is one source case in each cluster and the rest case (s) in a cluster is due to transmission (Small et al., 1994), we calculated the transmission rates of drug-resistant tuberculosis. However, it is apparent that the transmission events for clusters 13, 16, and 17 occurred before the development of drug-resistance in these isolates. In addition, in cluster 12, 12A and 12B might be due to one transmission event of a drug-resistant strain, whereas 12C through 12F obviously resulted from different drug-resistant transmission event(s). Therefore clusters 13, 16, and 17 were excluded from the calculation, and cluster 12 was treated as two clusters. After these adjustments, there were 25 drug-resistant isolates in 19 clusters were due to transmission. The overall transmission rate of drug-resistant tuberculosis was 10.9% (25/230), the transmission rate of drug-resistant Beijing isolates was 15% (21/140), and the transmission rate of drug-resistant non-Beijing isolates was 4.4% (4/90). These data suggest that the recent transmission of drug-resistant tuberculosis in Singapore was low and Beijing strains were more transmissible than were non-Beijing strains.

Table 5.2. Characteristics of clustered drug-resistant isolates

Cluster No.[a]	Genotypes	No. of IS*6110*	MIRU-VNTR patterns	Isolates[b]	Resistant patterns[c]			
					INH	RIF	STR	EMB
1	Beijing	20	223325163543	1A	R	R	R	S
				1B	R	R	R	S
2	Beijing	18	223325173533	2A	R	R	S	S
				2B	R	R	S	S
3	Beijing	22	222325173543	3A	S	S	R	S
				3B	S	S	R	S
4	Beijing	21	222325173533	4A	S	S	R	S
				4B	S	S	R	S
5	Beijing	22	223325173533	5A	R	R	S	S
				5B	R	R	S	S
6	Beijing	21	222325173543	6A	S	R	S	S
				6B	S	R	S	S
7	Beijing	20	222325153343	7A	S	S	R	S
				7B	S	S	R	S
8	Beijing	20	223325163533	8A	S	S	R	S
				8B	S	S	R	S
				8C	S	S	R	S
9	Beijing	20	223325143433	9A	S	S	R	S
				9B	S	S	R	S
10	Beijing	12	223325173433	10A	R	S	R	S
				10B	R	S	R	S
11	Beijing	13	223325173433	11A	S	S	R	S
				11B	S	S	R	S
12	Beijing	21	222325173533	12A	R	S	S	S
				12B	R	R	S	S
				12C	S	S	R	S
				12D	S	S	R	S
				12E	S	S	R	S
				12F	S	S	R	S
13	Beijing	19	203325173533	13A	R	S	S	S
				13B	S	S	R	S
14	Beijing	20	223325153543	14A	R	S	S	S
				14B	R	S	S	R
15	Beijing	15	223325173433	15A	R	S	R	S
				15B	S	S	R	S
				15C	S	S	R	S
				15D	S	S	R	S
				15E	S	S	R	S
16	Beijing	18	223325173533	16A	R	S	S	S
				16B	S	S	R	S
17	T	2	242325152322	17A	R	S	S	S
				17B	S	S	R	S
18	T	13	226326153227	18A	R	S	S	S
				18B	R	S	S	S
19	S	15	22n325173433[d]	19A	R	S	R	S
				19B	R	S	R	S
20	EAI	1	254326323513	20A	R	S	R	S
				20B	R	S	R	S
21	EAI	0	254326223513	21A	S	S	S	R
				21B	S	S	S	R

Notes to Table 5.2. [a]Clusters are numbered 1 through 21. [b]Isolates in each cluster are labeled with A, B, C, D, E, or F, after cluster number. [c]INH, isoniazid; RIF, rifampin; STR, streptomycin; EMB, ethambutol; R, resistant; S, sensitive. [d]n = 10.

5.3.4 Assessment of resistant pattern and Beijing genotype as clustering factors

The clustering risk of drug-resistant *M. tuberculosis* isolates with different resistant patterns and of Beijing isolates were examined (Table 5.3). INH-resistant (resistance to at least INH) isolates were inversely associated with clusters [OR (95% CI), 0.20 (0.10-0.38), $p < 0.001$]. When stratified by INH-monoresistance and INH-polyresistance/MDR, only INH-monoresistant isolates remained inversely associated with clusters [OR (95% CI), 0.25 (0.12-0.54), $p < 0.001$], whereas INH-polyresistant/MDR isolates were not associated with clusters [OR (95% CI), 0.70 (0.36-1.41), $p = 0.324$]. On the contrary, STR-resistant (resistance to at least STR) isolates were positively associated with clusters [OR (95% CI), 2.56 (1.35-4.86), $p = 0.004$]; when stratified by STR-monoresistance and STR-polyresistance, the association between STR-monoresistant isolates and clusters became stronger [OR (95% CI), 3.03 (1.59-5.80), $p < 0.001$], but no significant association between STR-polyresistant isolates and clusters was observed [OR (95% CI), 0.62 (0.26-1.43), $p = 0.263$]. Neither RIF-resistant isolates nor MDR were associated with clusters ($p > 0.05$). The drug-resistant isolates of the Beijing genotype were significantly associated with clusters [OR (95% CI), 3.20 (1.52-6.70), $p = 0.002$].

Multivariate logistic regression analysis was performed to adjust any potential confounding effect between Beijing isolates and STR-resistant or STR-monoresistant isolates because Beijing isolates were associated with STR-resistance or STR-monoresistance. After the adjustments, the association remained between Beijing

genotype strains and clusters no matter of being adjusted by STR-resistant isolates [OR (95% CI), 2.61 (1.19-5.73), $p = 0.017$] or by STR-monoresistant isolates [OR (95% CI), 2.42 (1.10-5.31), $p = 0.028$]. Similarly, although the associations remained between STR-resistant isolates and clusters as well as between STR-monoresistant isolates and clusters, Beijing isolates showed a negative adjustment to the association of STR-resistant isolates with clusters [OR (95% CI), 2.01 (1.02-3.96), $p = 0.044$], but a positive adjustment to the association between STR-monoresistant isolates and clusters [OR (95% CI), 3.43 (1.71-6.89), $p < 0.001$].

Table 5.3. Clustering risk of isolates with different resistant patterns and of Beijing isolates

Risk factors	Clustered isolates (n = 50)	Unique isolates (n = 180)	Univariate analysis[a]		Multivariate analysis[b]	
			OR (95% CI)	*p*	OR (95% CI)	*p*
Resistant patterns						
at least INH[e]	22	144	0.20 (0.10-0.38)	0.000		
INH-mono	9	84	0.25 (0.12-0.54)	0.000		
INH-poly&MDR	13	60	0.70 (0.36-1.41)	0.324		
at least STR	31	70	2.56 (1.35-4.86)	0.004	2.01 (1.02-3.96)	0.044
STR-mono	24	32	3.03 (1.59-5.80)	0.001	3.43 (1.71-6.89)	0.000
STR-poly	7	38	0.62 (0.26-1.43)	0.263		
at least RIF[e]	9	38	0.82 (0.37-1.81)	0.629		
MDR	7	32	0.75 (0.32-1.79)	0.529		
Beijing genotype	40	100	3.20 (1.52-6.70)	0.002	2.61 (1.19-5.73)[c] 2.42 (1.10-5.31)[d]	0.017 0.028

[a]χ^2 test. [b]Logistic regression. OR, odds ratio; CI, confidence interval. [c]Adjusted by resistance to at least STR. [d]Adjusted by monoresistance to STR. [e]Including the 7 MDR isolates in this table.

5.3.5 Relationships of drug-resistant phenotypes with *M. tuberculosis* genotypes

The relationships between drug-resistant phenotypes and *M. tuberculosis* genotypes were analyzed by comparing with the 364 drug-susceptible isolates which have been described in Chapter 2. As shown in Table 5.4, monoresistance to INH was inversely associated with Beijing isolates [OR (95% CI), 0.494 (0.31-0.788), p = 0.003] but positively associated with EAI isolates [OR (95% CI), 1.804 (1.099-2.961), p = 0.019]. However, when considered all the INH-resistant patterns (resistance to at least INH), INH-resistance was neither associated with Beijing isolates nor EAI isolates ($p > 0.05$). On the contrary, monoresistance to STR was positively associated with Beijing isolates [OR (95% CI), 4.571 (2.208-9.452), $p < 0.001$] but negatively associated with EAI isolates [OR (95% CI), 0.131 (0.035-0.499), p = 0.001]. STR-resistance remained positively associated with Beijing isolates [OR (95% CI), 3.306 (1.969-5.549) $p < 0.001$] and negatively associated with EAI isolates [OR (95% CI), 0.307 (0.145-0.649) p = 0.001] when considered all the STR-resistant patterns (resistance to at least STR). Either MDR [OR (95% CI), 2.657 (1.282-5.501), p = 0.008] or all RIF-resistant patterns [OR (95% CI), 2.883 (1.442-5.759), p = 0.002] was associated with Beijing isolates.

Table 5.4. Distribution of drug-resistant and drug-susceptible isolates by *M. tuberculosis* genotypes[a]

Resistant patterns	No. of isolates	No. of isolates (%) by genotypes							
		Beijing	EAI	CAS	S	Haarlem	LAM	T	X
Mono-resistance									
INH	93	34 (36.6)[b]	31 (33.3)[c]	-	7 (7.5)	7 (7.5)	2 (2.2)	12 (12.9)	-
STR	57	48 (84.2)[d]	2 (3.5)[e]	1 (1.8)	-	3 (5.3)	1 (1.8)	2 (3.5)	-
RIF	7	6 (85.7)	1 (14.3)	-	-	-	-	-	-
MDR & poly-resistance									
MDR	41	31 (75.6)[f]	4 (9.8)	-	1 (2.4)	2 (4.9)	-	3 (7.3)	-
Poly	34	23 (67.6)	5 (14.7)	1 (2.9)	3 (8.8)	1 (2.9)	-	1 (2.9)	-
Resistance to at least:									
INH	168	90 (53.6)	41 (24.4)	1 (0.6)	9 (5.4)	9 (5.4)	2 (1.2)	16 (9.5)	-
STR	103	82 (79.6)[g]	8 (7.8)[h]	2 (1.9)	3 (2.9)	4 (3.9)	1 (1.0)	3 (2.9)	-
RIF	48	37 (77.1)[i]	5 (10.4)	-	1 (2.1)	2 (4.2)	-	3 (6.3)	-
Drug-susceptible[j]	364	196 (53.8)	79 (21.7)	2 (0.5)	16 (4.4)	29 (8.0)	6 (1.6)	33 (9.1)	3 (0.8)

[a]Two monoresistant isolates to EMB were excluded, and comparison of frequencies between drug-resistant and drug-susceptible groups is by χ^2 or Fisher's exact test.
[b]OR (95% CI), 0.494 (0.31-0.788), $p = 0.003$.
[c]OR (95% CI), 1.804 (1.099-2.961), $p = 0.019$.
[d]OR (95% CI), 4.571 (2.208-9.452), $p < 0.001$.
[e]OR (95% CI), 0.131 (0.035-0.499), $p = 0.001$.
[f]OR (95% CI), 2.657 (1.282-5.501), $p = 0.008$.
[g]OR (95% CI), 3.306 (1.969-5.549), $p < 0.001$.
[h]OR (95% CI), 0.307 (0.145-0.649), $p = 0.001$.
[i]OR (95% CI), 2.883 (1.442-5.759), $p = 0.002$.
[j]Data from Chapter 2.

5.4. Discussion

5.4.1 Transmission of drug-resistant tuberculosis

Drug-resistant tuberculosis could result either from transmission (resistance among new cases) or from inadequate treatment (resistance among previously treated cases). Thus, a better understanding of the relative contribution of the two situations has practical implications for assessing and improving tuberculosis control programs. In this study, we have demonstrated that only about 11% of patients with drug-resistant tuberculosis were due to recent transmission. This observation suggests that recent transmission contributes only a small part to the incidence of drug-resistant tuberculosis in Singapore.

Since 1953, it has been recognized by animal studies that INH-resistant strains are less virulent than drug-susceptible strains as demonstrated by causing less disease in guinea pigs (Middlebrook and Cohn, 1953). Molecular epidemiological studies have shown that INH-resistant isolates were less likely to be in a cluster defined by DNA fingerprint in the Netherlands (van Soolingen et al., 1999) and to cause secondary cases in San Francisco (Burgos et al., 2003) than INH-sensitive ones. In the present study, we also found that INH-resistant isolates were less likely to be in a cluster compared to isolates with other resistant patterns. But this was restricted only to INH-monoresistant isolates not INH-polyresistant/MDR isolates. INH-polyresistant/MDR isolates were not related with clusters. Taken together, these findings suggest that INH-monoresistant strains have reduced transmissibility, perhaps because some INH-resistance conferring gene alterations reduce the virulence of the bacilli (Barnes and Cave, 2003).

In New York City (Friedman et al., 1995) and Poland (Sajduda et al., 2004), MDR strains were more likely to cluster. However, in Mexico (Garcia-Garcia et al.,

2000a, 2000b) and South African gold-mining community (Godfrey-Faussett et al., 2000), patients with MDR-TB were significantly associated with a decreased likelihood of being clustered. In San Francisco (Burgos et al., 2003), MDR-TB cases were not likely to produce new, incident drug-resistant tuberculosis cases. It is possible to be more likely in clusters if some individual MDR strains are high virulent. In the present study, no association was observed between MDR isolates and clusters. This might be due to our small MDR sample size or because our comparison was performed within drug-resistant population instead of between drug-resistant and drug-susceptible populations as the other studies did. This is because our drug-sensitive sample was collected in a short period (5 months) which is not long enough to reflect the extent of recent transmission (van Soolingen, 2001), thus not suitable for transmission analysis.

In addition to STR-monoresistant isolates, Beijing genotype isolates were also found to be more likely in a cluster than were non-Beijing isolates. This observation agrees with that of studies performed in Russia (Toungoussova et al., 2002, 2003). Because STR-resistance was strongly associated with Beijing genotype strains, multivariate analysis was then performed to adjust for any potential confounding effect between the two factors. Although these two risk factors for clustering remained independent from each other after multivariate analysis, because of as high as 84% of the STR-monoresistant isolates belonging to the Beijing genotype, it remains difficult to ascertain an independent role for STR-monoresistance in clustering.

5.4.2 *M. tuberculosis* genotypic preference to drug-resistant phenotypes

Although drug-resistant tuberculosis among previously treated cases results from man-made factors, increasing evidence suggest that drug-resistant phenotypes are not equally distributed among *M. tuberculosis* genotypes, suggesting that different *M. tuberculosis* genotypes may have different preference for developing drug-resistant phenotypes (Anh et al., 2000; Chan et al., 2001; Toungoussova et al., 2002, 2003; Baker et al., 2004; Jou et al., 2005). In this population-based study with a substantial number of resistance among previously treated cases, we demonstrated that there were significant positive/negative associations between *M. tuberculosis* genotypes and drug-resistant patterns. For example, INH-monoresistance was found to be more frequent in EAI strains but less often in Beijing strains; in contrast, STR-monoresistance occurred with a significant high proportion in Beijing strains, but less likely occurred in EAI strains.

So far the findings are discrepant on the relationship between INH-resistance and Beijing genotype strains. In Hong Kong, an inverse association between INH-resistance and Beijing genotype strains was observed (Chan et al., 2001), but in Vietnam (Anh et al., 2000) and in Taiwan (Jou et al., 2005), the association was found to be positive. In this study, the inverse association between INH-monoresistance and Beijing strains as well as the positive association between INH-monoresistance and EAI strains were resolved when considered all INH resistant patterns including MDR, suggesting that INH-polyresistance and MDR have introduced negative confounding effect to the significant associations. In supportive of this suggestion, the Hong Kong study (Chan et al., 2001) did not find significant association between Beijing genotype strains and MDR. This could be inferred that the confounding effect of INH-polyresistance and MDR were

at less extent compared to the present study in which a strong significant association between Beijing isolates and MDR was observed. The confounding effect suggests that the INH-monoresistant Beijing strains and the INH-polyresistant/MDR Beijing strains may represent different subgenotypes of the Beijing family. Baker et al. (2004) have provided supportive evidence that the INH resistance-conferring mutation *katG* ^{A944C} was positively associated with Delhi strains, and the *inhA* ^{C15T} promoter mutation positively associated with EAI strains.

However, studies performed in different settings have reported consistent results on the relationship of STR-resistance with Beijing genotype strains that Beijing strains are positively associated with STR-resistance (Anh et al., 2000; Toungoussova et al., 2002, 2003; Baker et al., 2004). This association was also detected in the present study.

The inconsistent or complex findings in INH-resistance and the consistent findings in STR-resistance may be related with their complexity of drug-resistance conferring mutations. INH-resistance has been found to be involved in multiple genes and mutations, but STR-resistance has been only related to two mutations of two genes and one of them is predominant (Ramaswamy and Musser, 1998). This has been the case for this study sample. Previous studies have shown that 64% of the INH-resistant isolates of the present study carried potential drug-resistance conferring mutations and/or deletions in genes *kat*G, *inh*A, *oxy*R, *ahp*C, *kas*A, and *ndh*, and the remaining 36% of isolates did not show any of the targeted genetic alterations in these genes (Lee et al., 1999, 2001). On the other hand, as high as 90% of our STR-resistant isolates carried drug-resistance conferring mutations only in the codon 43 (82%) and codon 88 (8%) of

*rps*L gene, and only 10% of isolates did not show the targeted mutations (Lee et al., unpublished data).

In summary, these data showed that recent transmission makes a small contribution to the incidence of drug-resistant tuberculosis in Singapore, suggesting a limited transmission of drug-resistant tuberculosis. The disequilibrium in distribution of resistant patterns among *M. tuberculosis* genotypes suggests that bacterial factor(s) also have a role to play in the development of drug-resistance.

CHAPTER 6

CLINICAL AND IMMUNOLOGICAL COMPARISON OF TUBERCULOSIS ASSOCIATED WITH *M. TUBERCULOSIS* BEIJING AND NON-BEIJING GENOTYPE STRAINS

6.1 Introduction

Molecular epidemiology has greatly advanced the understanding of population structure and geographic distribution of *Mycobacterium tuberculosis* worldwide. An important finding is the high prevalence of the *M. tuberculosis* Beijing genotype strains (Bifani et al., 2002; Glynn et al., 2002). The Beijing genotype was first described in 1995 because of its predominance in the Beijing area of China, the high similarity of IS*6110* RFLP patterns and the identical polymorphism pattern of the direct repeat region of *M. tuberculosis* genome between isolates (van Soolingen et al., 1995). Thereafter, it has been shown that strains of this genotype are predominant in other East Asian areas (Anh et al., 2000; Park et al., 2000; Chan et al., 2001; Sun et al., 2004) and prevalent all over the world (Bifani et al., 2002; Glynn et al., 2002). In Vietnam, it is an emerging pathogen associated with younger patients, and hence with active transmission (Anh et al., 2000). The selective advantage of the Beijing genotype has led to the postulation that it may be an "escape variant" of mass BCG vaccination (van Soolingen et al., 1995).

In addition to the wide dissemination and local predominance, Beijing strains have been reported to be associated with various phenotypes. The association with drug-resistance is the most frequently reported, such as in Vietnam (Anh et al., 2000), New York (Bifani et al., 1996), Cuba (Diaz et al., 1998), Estonia (Kruuner et al., 2001), and in Russia (Toungoussova et al., 2003). In addition, the Beijing genotype was also found to be associated with treatment failure and tuberculosis relapse in Vietnam (Lan et al., 2003), and with febrile response to treatment in Indonesia (van Crevel et al., 2001).

Two animal studies have been performed in mice comparing the infections associated with selected Beijing and non-Beijing strains (Manca et al., 2001; López et al.,

2003). In the study by Manca et al. (2001), the Beijing genotype strain HN878 was found to be hypervirulent. Mice infected with this strain failed to induce a Th1 immune response, as evidenced by lower levels of IFN-γ and TNF-α in the lungs than mice infected by other strains. In the study by López et al. (2003), mice infected with Beijing strains were characterized by extensive pneumonia, early but transient TNF-α expression, and higher bacillary loads in the lungs, and significantly higher earlier mortality. Expression of IFN-γ was faint, similar to the infection of *M. canettii* strains but lower than the infection of *M. tuberculosis* laboratory strain H37Rv.

All these findings suggest that the Beijing genotype clone of *M. tuberculosis* behaves differently from the others. However, there has been no study performed to compare the immune responses associated with infections caused by Beijing and non-Beijing strains in patients. *M. tuberculosis* Beijing strains accounted for >50% of tuberculosis cases and were associated with tuberculosis relapse and drug-resistance in Singapore (Chapters 2-5) and this is therefore a good study setting for comparison study of tuberculosis caused by Beijing and non-Beijing strains. In this prospective study, we aimed to determine whether there are clinical and biological differences in the host response to infections caused by Beijing and non-Beijing strains. In particular we hypothesized that Beijing strains elicit a weaker Th1 immune response, as shown by a reduced production of IFN-γ.

6.2 Patients and Methods

6.2.1 Patients and setting

Inpatients with pulmonary tuberculosis were prospectively and consecutively enrolled from the Departments of Infectious Diseases and General Medicine, Tan Tock Seng Hospital (TTSH), and the Department of Medicine, National University Hospital (NUH), from September 2004 to May 2005. Subject inclusion criteria were: (1) clinical presentation and radiographic findings considered to be strongly suggestive of pulmonary tuberculosis by the primary physicians, (2) ≥ 18 years old, (3) antituberculous treatment naïve or not longer than 48 hours, (4) able and willing to give informed consent. Patients who had a past history of tuberculosis, who were known to have HIV infection or other coexisting systemic diseases, such as chronic renal failure, diabetes mellitus, and neoplastic diseases, and pregnant or breastfeeding women were excluded. Approval for this study was granted by the Ethics Committee of the National Healthcare Group (NHG), Singapore. Written informed consent was obtained from all study subjects.

6.2.2 Demographic and clinical data collection.

Demographic, clinical, and routine laboratory testing data were collected by interview of patients or from medical records. Demographic characteristics included date of birth, place of birth, sex, and race. The data of BCG scar, BCG vaccination history, TB contact history, and risk factors for HIV infection were collected by interview of the subjects. The information of clinical symptoms and their durations, including cough (productive or not, sputum color), fever, night sweats, haemoptysis, weight loss, appetite loss, and chest pain, were first collected from medical records and confirmation of the information was sought during interview of patients. Routine temperature charts were examined covering the time of admission until the time of enrolment. Axillary temperature was recorded in

both hospitals using a digital thermometer every 6 hours. The patient was defined having fever if there was a recorded temperature of greater than 37.5 ^{0}C at any time. Routine laboratory test data including white blood cells, differential counts of white blood cells, serum hemoglobin, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), AFB smear results, bacillary culture and drug-susceptibility test results were collected from medical records. Anemia was defined when hemoglobin < 13 g/dL in males and < 11.8 g/dL in females. All patients were screened for risk factors of HIV infection and offered HIV serology testing.

6.2.3 Assessment of chest X-ray (CXR) presentation

Chest X-ray (CXR) films were reviewed at each institution by a single clinician experienced in the diagnosis and management of tuberculosis who were blinded to *M. tuberculosis* genotyping results. The disease extent on CXR was classified as: (1) unilateral disease, (2) bilateral disease, (3) cavitary disease, (4) pleural effusion, and (5) miliary disease.

6.2.4 DNA extraction from sputum

Sputum specimens were collected from each subject at enrolment. To decontaminate and liquefy sputum samples, 1 to 2 ml of sputum samples were incubated for 20 minutes with equal volume of 1% N-Acetyl-L-Cysteine (NALC)-2% NaOH, votexed every 5 minutes and then centrifuged at 14,000 rpm for 10 minutes. The supernatants were decanted, and the sediment was resuspended in 400 μl of digestion buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% Tween 20, pH8.0, containing 100 μg/ml of proteinase K). The

digestion was allowed to proceed overnight at 56 ^{0}C with rigorous shaking. After the digestion, DNA was extracted using phenol-chloroform/isoamyl alcohol, precipitated with absolute ethanol and washed with 70% ethanol according to standard protocol (Moore and Dowhan, 1998). DNA was finally dissolved in 50 µl distilled water.

To assess the integrity of the extracted DNA and the presence/absence of PCR inhibitor, 2 µl DNA samples were analyzed using PCR which amplifies 268-bp human genomic globin DNA with forward primer 5'-GAA GAG CCA AGG ACA GGT A-3' and reverse primer 5'-CAA CTT CAT CCA CGT TCA CC-3'. Samples which were positive for the amplicon were stored at -20 ^{0}C for *M. tuberculosis* genotyping as described below, samples which were negative for the amplicon were re-extracted and purified as described above.

6.2.5 Genotyping of *M. tuberculosis*

M. tuberculosis was genotyped using spoligotyping and MIRU-VNTR typing as described in Chapter 2 except that DNA sample for PCR amplification was 2 µl of the above DNA extraction for each PCR reaction and the MIRU-VNTR allele number was manually converted according to amplicon size.

6.2.6 Isolation of plasma

Fifteen ml of venous blood samples were collected using heparinized tubes. The blood samples were centrifuged at 300x g for 10 minutes to separate plasma and blood cells. Upper phase plasma was recovered and further centrifuged at 14,000 rpm for 15 minutes

at 4 ^{0}C using a refrigerated centrifuge. Plasma was then recovered and aliquoted in 0.5 ml of volume and stored in -80^{0}C freezer until use.

6.2.7 Isolation of PBMC

After removal of plasma as described above, the precipitated blood cells were gently resuspended in appropriate volume of RPMI 1640 Media to make the final volume to 12 ml. Added 6 ml of Ficoll-Paque Plus media (Amersham) to each of two 15 ml centrifuge tubes and carefully laid (do not disturb the Ficoll-Paque surfaces) 6 ml of the blood cells onto each of the Ficoll-Paque layers. The tubes were centrifuged at 400x g for 40 min at room temperature using swing rotor without brake. After centrifuge, the upper liquid phases were carefully removed and the buffy layers (leukocytes) were recovered into a fresh 15 ml centrifuge tube. Appropriate volume of RPMI 1640 was added to make the final volume to 12 ml and the cells were gently mixed by inversion and then equally divided into two centrifuge tubes. After centrifuge at 300x g for 15 minutes, the precipitated cells were resuspended in 2 ml of RPMI 1640 and then pooled together for a further centrifuge at 300x g for 15 min. The final cell palette was resuspended in 1 ml of RPMI 1640. Fifty μl of the cell suspension was diluted in 450 μl of Trypan Blue for cell counting using a haemocytometer. The cells were aliquoted at 2 x10^{6} cells for RNA isolation.

6.2.8 Cytokine ELISA

Plasma level of IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-18, TGF-β1, and TNF-α were measured using quantitative ELISA kits (Bender MedSystems, Austria).

The detection limits of the above ELISA kits were 0.66 pg/ml (kit *BMS228INST*) (IFN-γ), 2.3 pg/ml (kit *BMS221INST*) (IL-2), 0.66 pg/ml (kit *BMS225INST*) (IL-4), 1.45 pg/ml (kit *BMS278INST*) (IL-5), 0.92 pg/ml (BMS213INST) (IL-6), 0.66 pg/ml (kit *BMS215INST*) (IL-10), 0.93 pg/ml (kit *BMS238INST*) (IL-12), 0.99 pg/ml (kit *BMS231INST*) (IL-13), 9.2 pg/ml (kit *BMS267/2*) (Il-18), 23.76 pg/ml (kit *BMS249/2*) (TGF-β1), and 1.65 pg/ml (kit *BMS223INST*) (TNF-α), respectively. A high sensitivity ELISA kit (*BMS228HS*) for IFN-γ from the same manufacturer was used to measure some samples with very low level of IFN-γ. The sensitivity of this kit was 0.06 pg/ml. The manufacturer's protocols were followed and each sample was assayed in duplicate. Absorbance was read using an *anthos* 2020 ELISA reader (Anthos Labtech Instruments, Austria) at wavelength 450 nm and 620 nm was used as reference wavelength.

6.2.9 Total RNA isolation from

Total RNA isolation from PBMC was performed using TRIZOL® reagent (Invitrogen) according to the manufacturer's protocol. Cells (2 x 10^6 cells) were lysed in 1 ml of TRIZOL® reagent by repetitive pipetting. The lysed cell samples were incubated for 5 minutes at room temperature (RT) to permit the complete dissociation of nucleoprotein complexes. Following the incubation, 0.2 ml of chloroform was added to the tubes and vigorously shook the tubes for 15 seconds with hand. After incubation 2-3 minutes at RT, the tubes were centrifuged at 12,000x g for 15 minutes at 4^0C to separate RNA containing phase. After the centrifuge, the upper RNA containing supernatant was recovered into a fresh tube and RNA was precipitated by incubation with 0.5 ml of isopropyl alcohol for 10 minutes at RT. The tubes were then centrifuged at 12,000x g for 10 minutes at 4^0C to

pellet RNA. The RNA pellets were washed with 1ml of 75% ethanol and centrifuged again at 7,500x g for 5 minutes at 4^0C. After removal of the ethanol, the RNA samples were briefly air-dried and dissolved in 10 μl of RNase free water. The purity and quantity of the RNA samples were measured using spectrophotometer at wavelength 260 nm and 280 nm.

6.2.10 cDNA synthesis by RT-PCR

cDNA was reverse transcribed from total RNA using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche). Briefly, 0.5 to 1 μg of total RNA was incubated with 1x reaction buffer, 5 mM of $MgCl_2$, 1 mM dNTP, 1.6 μg (2.0 μl) of oligo-$p(dT)_{15}$ primer, 50 units (1μl) of RNase inhibitor, ≥ 20 units (0.8 μl) of AMV reverse transcriptase in a final volume of 20 μl at 25^0C for 10 minutes and then at 42^0C for 60 minutes. Followed the incubation, the AMV reverse transcriptase was inactivated by incubation the reaction at 99^0C for 5 minutes. All the incubations were performed on a thermocycler (Biometra, Germany). The resulting cDNA was diluted to a final volume of 200-500 μl with PCR-grade water. A negative control without RNA was performed in parallel.

6.2.11 Quantification of cDNA by Real-Time PCR

The cDNA of reverse-transcribed cytokine genes was relatively quantified using quantitative real-time PCR. The real-time PCR primers and standards for cytokine genes IFN-γ, IL-2, IL-4, IL-13, and IL-18, and for housekeeping gene β-actin were commercially obtained from Search-LC GmbH (Heidelberg, Germany). All the primers are located on exons, thus do not amplify potential contaminating genomic DNA. Real-

time PCR amplification was performed using the LightCycler™ FastStart Master Sybr®Green I kit (Roche) on the Roche LightCycler apparatus. Quantitative analysis was performed with the LightCycler software (version 3.5). Background fluorescence was removed manually by setting a noise band at crossing line = 0.6, quantification was based on log-linear standard curve. Melting curve analysis was performed to confirm the identity and specificity of the PCR products. All cytokine cDNA copies were standardized to the housekeeping gene β-actin. Negative control without any cDNA was performed in each real-time PCR run.

6.2.12 Statistical analysis

χ^2 test and Fisher's exact test were used to analyze categorical variables. Student's *t* test was used to compare normally distributed continuous variables and those variables which were normally distributed after log-transformation. Mann-Whitney test was used to compare continuous but not normally distributed variables. Multivariate logistic regression was used to adjust for potential confounding factors. A *p* value of <0.05 was considered statistically significant.

Sample size was calculated using parameters from the study of Verbon et al (1999), which gave a mean and standard deviation of IFN-γ levels of 29 pg/ml and 52 pg/ml respectively. Based on the difference in mean IFN-γ levels of patients with and without fever, we expect that greater than 100% difference in mean IFN-γ levels between Beijing and non-Beijing strains would be biologically meaningful. Therefore, with a power of 0.8 and two-sided level of significance of less than 0.05, the required sample size is 47 patients per group.

6.3 Results

6.3.1 Patient enrolment and determination of *M. tuberculosis* genotypes

A total of 54 patients were enrolled in this study (35 from TTSH and 19 from NUH) between September 2004 and March 2005. Recruitment was discontinued prior to completion of the originally planed sample size of 94 patients due to the combination of slower-than-projected enrollment, investigator fatigue, and the limiting constraints of study funding and time. One of the 54 patients was found to be HIV-infected, and was excluded from further analysis. Of the remaining patients, 16 tested negative for HIV antibodies. Those who were not tested did not have any apparent risk factors for HIV infection nor any evidence of immune suppression noted on physical examination. Two further patients were excluded because they were unable to provide a sputum specimen for genotyping. Nine patients were culture negative, smear negative and did not have *M. tuberculosis* detected by either spoligotyping or MIRU-VNTR typing. It is uncertain whether these patients had tuberculosis, and given that it is not possible to determine the genotype, they could not contribute to addressing the study hypothesis and were therefore excluded from further analysis.

For the 42 patients with culture-proven pulmonary tuberculosis, all had mycobacteria that were fully susceptible to rifampicin, streptomycin, isoniazid, and ethambutol. Of these 42, 38 (90.5%) were smear positive. *M. tuberculosis* DNA was detected in the sputum sample and successfully typed by MIRU-VNTR typing in 41 cases, and by spoligotyping in 39 cases. All the cases of failure of genotyping were also smear negative. The single case that could not be typed by either method was excluded

from further analysis. Therefore, the study analysis concerns 41 patients with genotyping data, all of whom had culture-proven tuberculosis.

Twenty-one (51.2%, 21/41) patients were infected with Beijing strains. As shown in Fig. 6.1, 19 isolates had the typical Beijing spoligotype which is absent of spacers 1-34 but present of spacers 35-43 (Filliol et al., 2002; Kremer et al., 2004). One isolate (N04) showed Beijing-like spoligotype which has been demonstrated also belonging to the Beijing lineage (Kremer et al., 2004). Although the isolate N07 was not detected by spoligotyping, MIRU-VNTR typing showed that it had a typical cluster MIRU-VNTR pattern (223325173533) specific for Beijing strains. The MIRU-VNTR locus 31 of the isolate T38 had two different allele numbers, possibly suggesting mixed infection by two Beijing strains.

Based on the spoligotypes and/or MIRU-VNTR patterns, the remaining 20 isolates were assigned to non-Beijing genotypes: the Haarlem family (6 isolates, 14.6%), the Latin American and Mediterranean (LAM) family (1 isolate, 2.4%), the T family (4 isolates, 9.8%), and the ancestral East-Africa-Indian (EAI) family (9 isolates, 22%). The isolate N01 was assigned to the EAI family based on its MIRU-VNTR pattern and the allele number of its locus 24, nearly all EAI strains have ≥ 2 allele copies in this locus (Supply et al., 2001; Sun et al., 2004a).

6.3.2 Demographic and epidemiological characteristics

Table 6.1 presents the demographic and epidemiologic characteristics of the subjects by *M. tuberculosis* Beijing and non-Beijing genotypes. The distributions of sex, ethnicity, and age did not differ significantly between the two groups of patients. The frequency of

Isolates			MIRU-VNTR Patterns										Spoligotypes	Genotypes
	2	4	10	16	20	23	24	26	27	31	39	40		
N07	2	2	3	3	2	5	1	7	3	5	3	3	ND	Beijing
N08	2	2	3	3	2	5	1	7	3	5	3	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T21	2	2	3	3	2	5	1	7	3	5	3	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T32	2	2	3	3	2	5	1	7	3	5	3	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T38	2	2	3	3	2	5	1	7	3	4/5	3	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T41	2	2	3	3	2	5	1	7	3	5	3	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T44	2	2	3	3	2	5	1	7	3	5	3	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T45	2	2	3	3	2	5	1	7	3	5	3	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T47	2	2	3	3	2	5	1	7	3	5	3	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T49	2	2	3	3	2	5	1	7	3	5	3	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T55	2	2	3	3	2	5	1	7	3	5	3	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
N14	2	2	3	3	2	5	1	7	3	3	3	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T51	2	2	3	3	2	5	1	7	3	4	3	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T18	2	2	3	3	2	5	1	9	3	5	3	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
N05	2	2	3	3	2	5	1	5	3	5	1	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T53	2	2	3	3	2	5	1	6	3	5	3	4	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
N13	2	2	2	3	2	5	1	7	3	5	4	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T11	2	2	2	3	2	5	1	7	3	5	4	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T33	2	2	3	3	2	5	1	7	1	5	3	1	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
T52	2	3	3	2	2	5	1	7	3	4	4	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	Beijing
N04	2	2	2	3	2	5	1	7	3	5	4	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■	Beijing
T34	2	2	2	3	2	5	1	7	3	5	10	3	■□□□□□□□□□□□□□□□□□□□□□■■■□□□□□□□□□□□■□□□□□	Haarlem
T03	2	2	2	3	2	5	1	4	3	3	2	3	■□■■□■■■■□■■■■□■■■■■■■■■□□□□□□□□□□□□□■■□■■□■	Haarlem
N26	2	2	2	3	2	5	1	5	3	3	2	3	■■■■■■■■■□■■■□□□□□■■■■■■■□□□■■■□■□□□□■■■■■■■	Haarlem
T50	2	2	6	3	2	5	1	5	3	3	2	3	■□□■■■■■■■■■■■■■■■■■■■■■■□□□□□□□■□□□□■■■■■■■	Haarlem
T12	1	2	2	3	2	5	1	5	3	4	2	3	■■■■■■■■□□□□□□□□□■□■■■■■□□□□□□□■□□□□■■■■■■■	Haarlem
T19	2	2	2	4	2	5	1	5	3	3	2	4	■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	Haarlem
N22	2	2	4	3	2	6	1	4	3	3	2	7	■■■■■■■■■■■■■■■■■■■■■□□□□■■□■■■■■□□□□■■■■■■■	LAM
T37	2	2	5	1	2	5	1	1	3	3	2	2	■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■■□□□□■■□□□□□□	T
T36	2	2	4	2	2	6	1	2	3	3	2	3	■■■■■■■■■□■■■■■■■■□■□□■■■■□■■■■■□□□□■■■■■■■	T
N28	2	4	2	3	2	5	1	5	2	3	1	2	■■■■■■■■■□■■■■□■■■■■■■■■□□□■■■■■□□□□■■□□■□■	T
T42	2	3	2	3	2	5	1	4	2	3	2	2	■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	T
T24	2	5	5	3	2	6	2	2	2	5	1	3	■□■■■■■■■□■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■	EAI
T35	2	5	4	3	2	6	2	2	3	4	3	2	■■□■■■■■■□■■■■■■■■■□□■■■■■■■□□□□■□■■■■■■■■	EAI
T30	2	7	4	3	2	6	2	2	3	4	3	2	■■□■■■■■■□■■■■■■■■■□□■■■■■■■□□□□■□■■■■■■■■	EAI
T54	2	5	4	3	2	6	2	2	3	4	3	2	■■□■■■■■■■■■■■■■■■■□□■■■■■■■□□□□■□■■■■■■■■	EAI
N09	2	5	4	2	2	5	2	2	3	5	2	2	■□□■■■■■■■■■■■■■■■■□□■■■■■■■□□□□■□■■■■■■■■	EAI
T02	2	5	4	3	2	6	2	2	3	4	3	4	■□□■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□■■■■	EAI
T40	2	5	4	3	2	6	2	2	3	4	3	2	□□□□□□□□□□□□□□□□□□□□□□□□■■■□□□□■□■■■■■■■■	EAI
T48	3	6	4	2	2	5	2	2	3	5	3	3	■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□□□□□□■■	EAI
N01	2	6	4	3	2	6	2	2	3	4	3	2	ND	EAI

Fig. 6.1 (page105). *M. tuberculosis* genotyping results of the 41 sputum specimens. *M. tuberculosis* isolates in the 41 sputum specimens were typed by spoligotyping and MIRU-VNTR typing. Spoligotyping generated 39 spoligotypes, MIRU-VNTR generated 41 patterns. Based on the spoligotypes and/or MIRU-VNTR patterns, each isolate was assigned to one of the *M. tuberculosis* genotypes. Beijing, Beijing genotype; Haarlem, Haarlem genotype; LAM, Latin American and Mediterranean genotype; T, T genotype; EAI, East-African-Indian genotype. ND, not detected.

Table 6.1. Characteristics of patients by *M. tuberculosis* genotypes[a]

Characteristics	Beijing n = 21 (%)	Non-Beijing n = 20 (%)	*P*
Male sex	14 (66.7)	10 (50.0)	0.297
Ethnicity			0.286
Chinese	14 (66.7)	12 (60.0)	
Malay	7 (33.3)	6 (30.0)	
Indian	0 (0)	2 (10.0)	
Age (y)			0.325
≤ 30	4 (19.0)	6 (30.0)	
31-59	12 (57.1)	11 (55.0)	
≥ 60	5 (23.8)	3 (15.0)	
BCG			0.303
Yes	11 (52.4)	13 (65.0)	
No/unknown	10 (47.6)	7 (35.0)	
Sputum Smear			0.486
Positive	19 (90.5)	19 (95.0)	
Median scores	3+	3+	
Known contact history	2 (9.5)	2 (10.0)	0.959
Residentships			0.141
Singapore resident[b]	17 (80.9)	12 (60.0)	
Foreigner	4 (19.1)	8 (40.0)	
Hospitals			0.796
TTSH	15 (71.4)	15 (75.0)	
NUH	6 (28.6)	5 (25.0)	

[a]Data shown are absolute numbers with percentage of the group total in brackets. Comparison of frequencies between groups is by χ^2 or Fisher's exact test.
[b]Citizens and permanent residents

past BCG vaccination did not differ between the two groups, and the proportion of smear positive cases was similar in each group. The median score for the positive smears was 3+ in each group. Four patients had known contact history with family members (2 in each group). The proportions of Beijing and non-Beijing genotype isolates were similar at the two hospitals.

6.3.3 Clinical and radiological features

Table 6.2 shows the clinical and chest radiographic manifestations of the patients by Beijing and non-Beijing genotypes. Cough was the most common symptom for the pulmonary tuberculosis patients, 38 of the 41 (92.7%) patients had cough. The less frequent manifestations were fever (26/41, 63.4%), and weight loss (28/41 68.3%). The frequencies of hemoptysis, appetite loss, night sweats, and chest pain were even lower.

Fever was recorded in 9 (42.9%) of the 21 patients infected with Beijing strains and 17 (85.0%) of the 20 patients infected with non-Beijing strains [OR (95% CI), 0.13 (0.03-0.57), $p = 0.005$]. In addition to fever, night sweats was also found to be less often in patients infected with Beijing genotype strains [OR (95% CI), 0.20 (0.04-0.99), $p = 0.049$]. To further test these associations, we performed multivariate logistic regression analysis. Independent variables included sex, age, and ethnicity, though they did not differ significantly between the two groups, as well as fever and night sweats. The inverse association between the Beijing strains and febrile response remained strong after multivariate adjustment [OR (95% CI), 0.12 (0.02-0.63), $p = 0.008$], suggesting that the Beijing genotype is an independent factor associated inversely with production of fever.

Table 6.2. Clinical and chest X-ray manifestations of patients by *M. tuberculosis* genotypes

Parameters	Beijing n = 21 (%)	Non-Beijing n = 20 (%)	Univariate analysis[a] OR (95% CI)	*p*	Multivariate analysis[b] OR (95% CI)	*p*
Clinical presentations						
Cough	18 (85.7)	20 (100)	0.00 (0.00-1.27)	0.125		
Median duration (range)[c]	60 (14-1440)	30 (2-180)		0.048		
Fever	9 (42.9)	17 (85.0)	0.13 (0.03-0.57)	0.005	0.12 (0.02-0.63)	0.008
Hemoptysis	4 (19.0)	6 (30.0)	0.55 (0.14-2.22)	0.414		
Weight loss	16 (76.2)	12 (60.0)	2.13 (0.58-7.87)	0.265		
Appetite loss	6 (28.6)	4 (20.0)	1.60 (0.40-6.39)	0.523		
Night sweats	2 (9.5)	7 (35.0)	0.20 (0.04-0.99)	0.049	0.20 (0.02-1.75)	0.145
Chest pain	2 (9.5)	4 (20.0)	0.42 (0.08-2.29)	0.343		
CXR presentations[d]						
Unilateral disease	7 (33.3)	8 (40.0)	0.75 (0.22-2.61)	0.658		
Bilateral disease	13 (61.9)	11 (55.0)	1.33 (0.39-4.52)	0.654		
Pleural effusion	1 (4.8)	1 (5.0)	0.95 (0.09-9.75)	0.972		
Cavity	2 (9.5)	7 (35.0)	0.20 (0.04-0.99)	0.049		
WBC count (cells/μl)						
Total WBC (mean ± SD)[e]	8629 ± 3331	10415 ± 3996		0.150		
Lymphocytes (mean ± SD)	1376 ± 614	1563 ± 983		0.496		
Monocytes (mean ± SD)	656 ± 340	901 ± 361		0.014		
Eosinophils (mean ± SD)	144 ± 194	80 ± 82		0.193		
Basophils (mean ± SD)	32 ± 17	40 ± 43		0.474		
Anemia[f]	14 (66.7)	13 (65.0)	1.08 (0.31-3.81)	0.910		
Hemoglobin (g/dL)						
Male (mean ± SD)	11.8 ± 1.2	11.3 ± 1.6				
Female (mean ± SD)	11.6 ± 1.9	11.3 ± 1.4				

Notes to Table 6.2. [a]χ^2 or Fisher's exact test were used for the categorical variables and Student's was used for the WBC counts after they were log-transformed; OR, odds ratio; CI, confidence interval. [b]Logistic regression. [c]Unit is in day. [d]Cavity is not exclusive from the other three entries. [e]SD, standard deviation. [f]Hemoglobin < 13 g/dL in males and < 11.8 g/dL in females.

However, the inverse association between night sweats and the Beijing strains was no longer significant after the multivariate adjustment [OR (95% CI), 0.20 (0.02-1.75), p = 0.145].

There was no significant difference in the frequency of cough between Beijing and non-Beijing infected patients (p = 0.125). However, in the 18 patients who had cough and were infected with Beijing strains, the duration of cough before the diagnosis varied widely from 14 days to 1440 days (4 yrs) with a median of 60 days, only approximately 39% (7/18) of such patients were identified and diagnosed within 1 month. While in the patients infected with non-Beijing strains, the range of the duration was much narrower, from 2 days to 180 days with a median of 30 days; much higher proportion (65%, 13/20) of the patients was identified within 1 month. The individual cough duration is shown in Fig. 6.2. As the cough duration data had too many ties (identical numbers) hence, the non-parametric Mann-Whitney U test does not fit this data. The data were therefore log-transformed and analyzed by *t* test. The difference between the two groups is statistically significant (p = 0.048). Surprisingly, there were two patients in the Beijing group might be as infectious foci for 4 years, because no other obvious cause was found for their cough. This finding suggests that patients infected with Beijing strains have longer duration of symptoms before being identified/diagnosed.

The CXR abnormalities associated with infections of Beijing and non-Beijing strains were compared (Table 6.2). There was significantly less cavitary disease in patients infected with Beijing strains [OR (95% CI), 0.20 (0.04-0.99), p = 0.049]. There

was no difference in the frequency of unilateral non-cavitary disease, bilateral non-cavitary disease, and pleural effusion. We did not find miliary disease in our subjects.

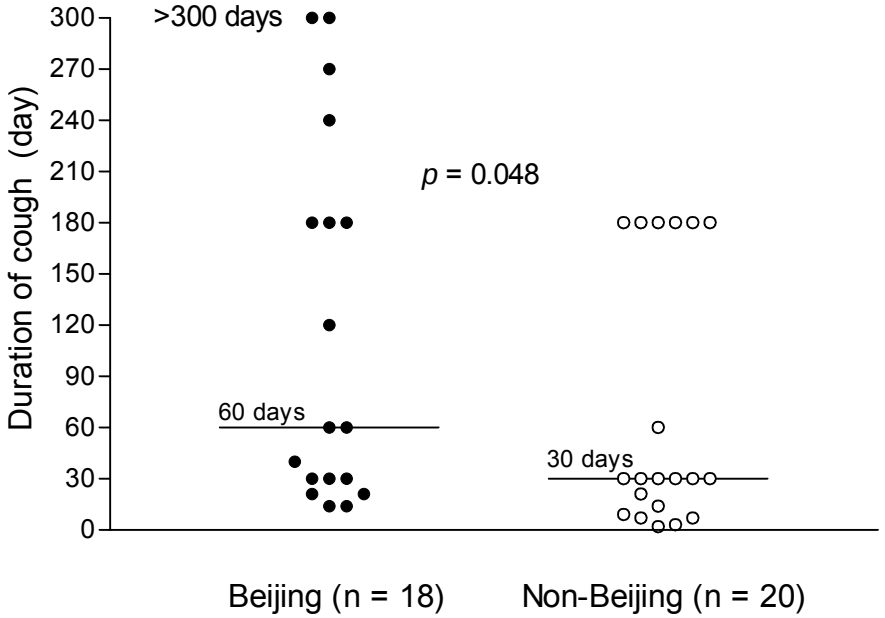

Fig. 6.2. Cough duration (day) of patients infected with Beijing and non-Beijing strains. Cough durations of the pulmonary tuberculosis patients before diagnosis were compared by Beijing and non-Beijing groups. Patients infected with Beijing strains had significantly longer cough (median, 60 days) compared to those infected with non-Beijing strains (median, 30 days). The two patients who had coughed for 4 years before diagnosis are expressed as > 300 days in the Beijing group. The two groups differed significantly ($p = 0.048$).

6.3.4 Laboratory parameters

The frequency of anemia in total was 65.9% (27/41), and there was no difference between the two groups (Table 6.2). Table 6.2 also presents total and differential counts of white blood cells of the patients. The mean counts for total white blood cells, lymphocytes, eosinophils, and basophils were not significantly different in each group. The mean monocyte count was significantly higher in non-Beijing group (mean, 901/μl)

compared to Beijing group (mean, 656/μl) (p = 0.014). Few patients had CRP and ESR tests done, and given the paucity of data we have not analyzed it further.

6.3.5 Plasma cytokine levels in Beijing and non-Beijing genotypes

Of the cytokines analyzed, only IFN-γ, IL-6, IL-18, and TGF-β1 were detectable in plasma. There was no statistically significant difference in the mean IFN-γ levels between patients infected with Beijing and non-Beijing strains (20.5 pg/ml, SD = 28.6 versus 33.9 pg/ml, SD = 95.2, p = 0.994) (Fig. 6.3). The mean plasma level of IL-6 was 13.2 pg/ml (SD = 14.7) in patients infected with Beijing strains and 11.1 pg/ml (SD = 15.4) in patients infected with non-Beijing strains; the difference was not significant (Fig. 6.4). The mean plasma level for IL-18 and TGF-β1 was respectively 409.9 pg/ml (SD = 376.1) and 99.7 ng/ml (SD = 72.8) in Beijing group, and 598.5 pg/ml (SD = 688.7) and 111.4 ng/ml (SD = 58.4) in non-Beijing group. Similarly, the differences were not significant (Figs. 6.5 and 6.6).

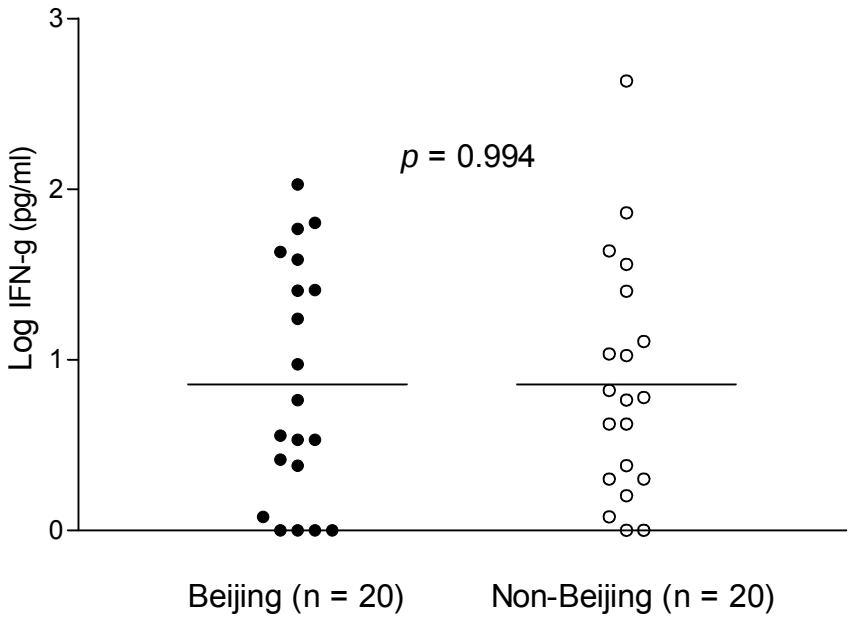

Fig. 6.3. Plasma levels of IFN-γ in pulmonary tuberculosis patients. Plasma level of IFN-γ in pulmonary tuberculosis patients was quantified using ELISA assay. Patients infected with Beijing and non-Beijing strains had similar mean IFN-γ level (p = 0.994). Bars indicate the log mean levels in each group.

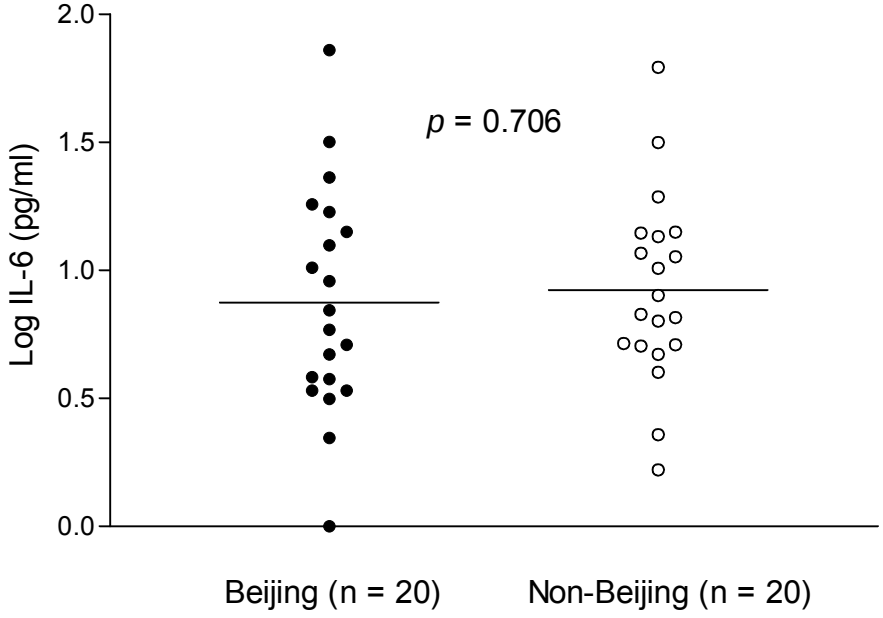

Fig. 6.4. Plasma levels of IL-6 in pulmonary tuberculosis patients. Plasma level of IL-6 in pulmonary tuberculosis patients was quantified using ELISA assay. Patients infected with Beijing and non-Beijing strains had similar mean IL-6 level (p = 0.706). Bars indicate the log mean levels in each group.

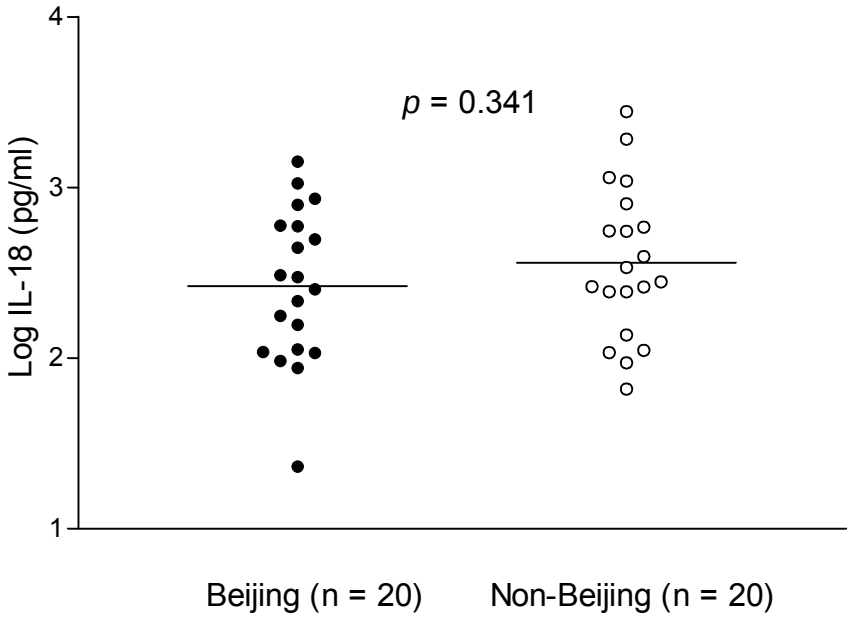

Fig. 6.5. Plasma levels of IL-18 in pulmonary tuberculosis patients. Plasma level of IL-18 in pulmonary tuberculosis patients was quantified using ELISA assay. Patients infected with Beijing and non-Beijing strains had similar mean IL-18 level (p = 0.341). Bars indicate the log mean levels in each group.

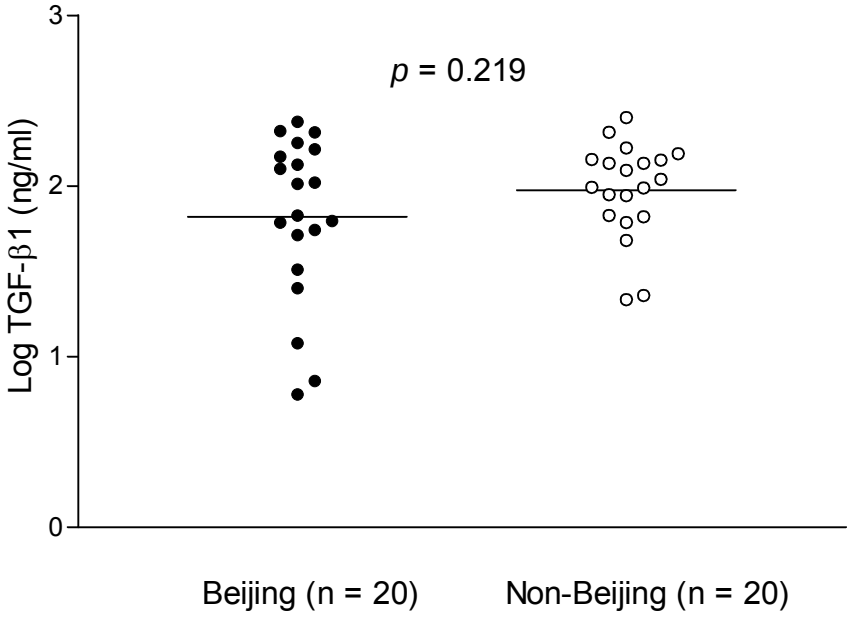

Fig. 6.6. Plasma levels of TGF-β1 in pulmonary tuberculosis patients. Plasma level of TGF-β1 in pulmonary tuberculosis patients was quantified using ELISA assay. Patients infected with Beijing and non-Beijing strains had similar mean TGF-β1 level (p = 0.219). Bars indicate the log mean levels in each group.

6.3.6 Cytokine gene expression analysis

We further analyzed the cytokine gene expression of PBMCs of the patients. Of the 40 blood samples collected and prepared for cytokine gene expression analysis, the first 15 samples had very low RNA yields, thus the cDNA transcribed from these samples was too low to be reliably quantified. The results presented below pertain to the 25 samples in which satisfactory RNA samples were obtained. The 25 samples were from 12 patients infected with Beijing strains (8 males, 66.7%; mean age ± SD = 52 ± 14) and 13 patients infected with non-Beijing strains (5 males, 38.5%; mean age ± SD = 48 ± 21). These characteristics were similar between the subgroup patients and the whole group of study subjects (refer to Table 6.1).

The gene expression of type 1 cytokines IFN-γ, IL-2 and IL-18, and type 2 cytokine IL-4 and IL-13 was quantitatively measured, and standardized as per 10^5 β-actin cDNA copies. The expression level of IL-13 was too low to be detected by the quantitative real-time PCR. The cDNA copies of IFN-γ, IL-2 and IL-18 did not differ significantly between the Beijing and non-Beijing groups (Figs. 6.7 to 6.9). The median cDNA copies for IFN-γ, IL-2 and IL-18 were respectively 232 (range, 77-691), 7 (range, 0-78), and 314 (range, 120-715) in patients infected with Beijing strains, and 228 (range, 39-491), 10 (range, 0-30), 355 (range, 81-1067) in patients infected with non-Beijing strains. The cDNA copies of IL-4 were significantly higher in non-Beijing strain (median = 26, range, 9-87) versus Beijing strain (median = 16, range, 0-68) infected patients (p = 0.018) (Fig. 6.10).

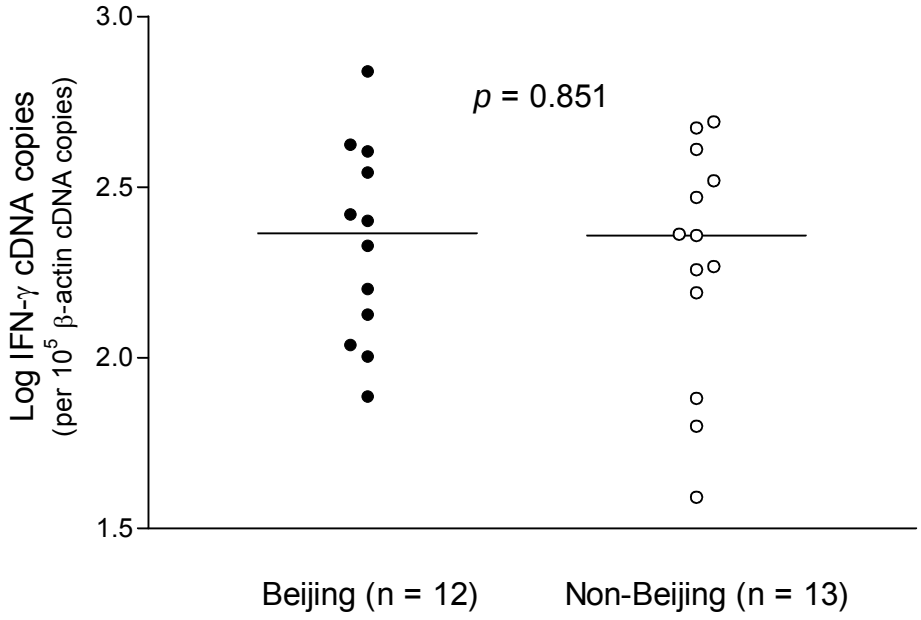

Fig. 6.7. cDNA copies of IFN-γ. Patients infected with Beijing and non-Beijing strains had similarly expressed cDNA copies of IFN-γ ($p = 0.851$). Log medians of cDNA copies are indicated by bars.

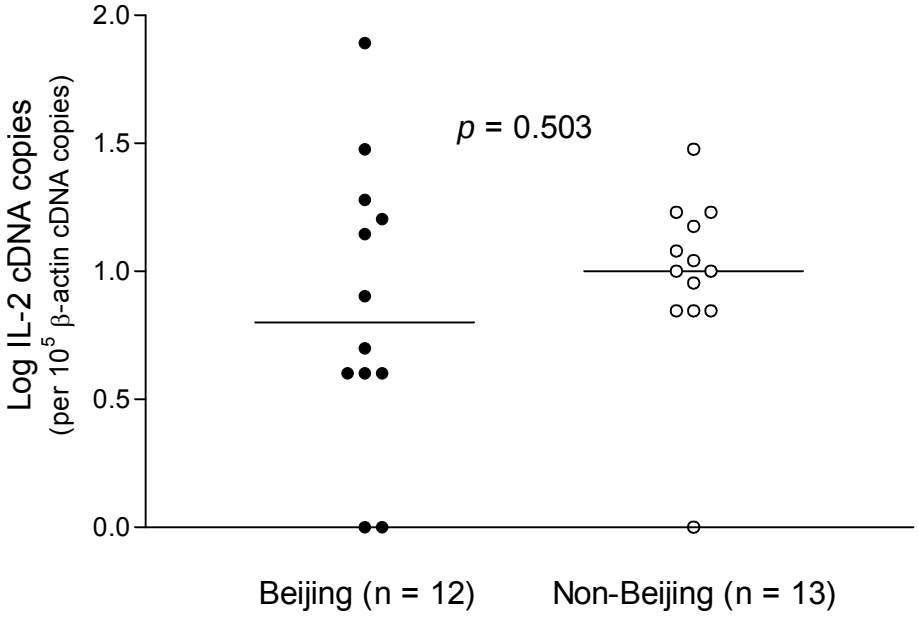

Fig. 6.8. cDNA copies of IL-2. Patients infected with Beijing and non-Beijing strains had similarly expressed cDNA copies of IL-2 ($p = 0.503$). Log medians of cDNA copies are indicated by bars.

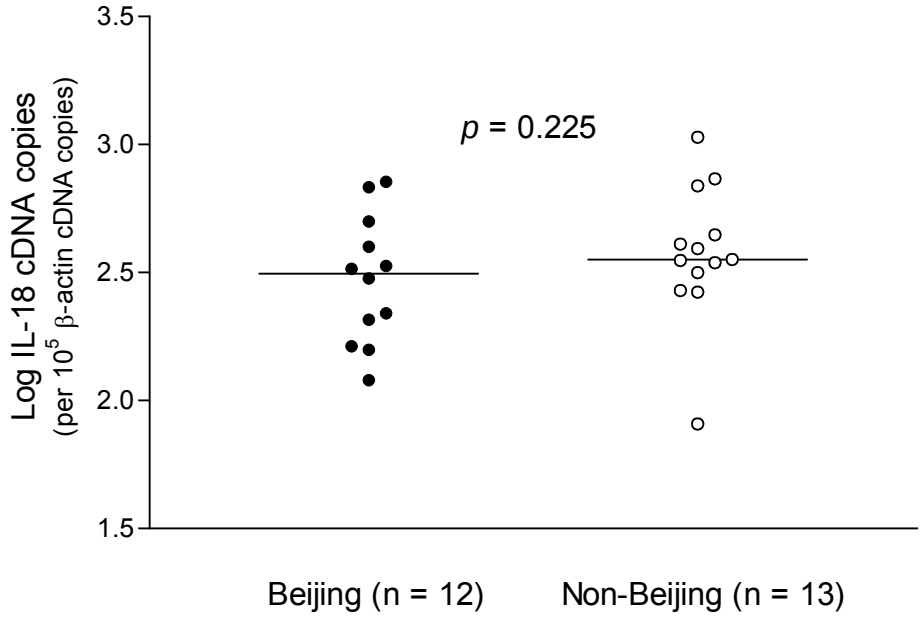

Fig. 6.9. cDNA copies of IL-18. Patients infected with Beijing and non-Beijing strains had similarly expressed cDNA copies of IL-18 ($p = 0.225$). Log medians of cDNA copies are indicated by bars.

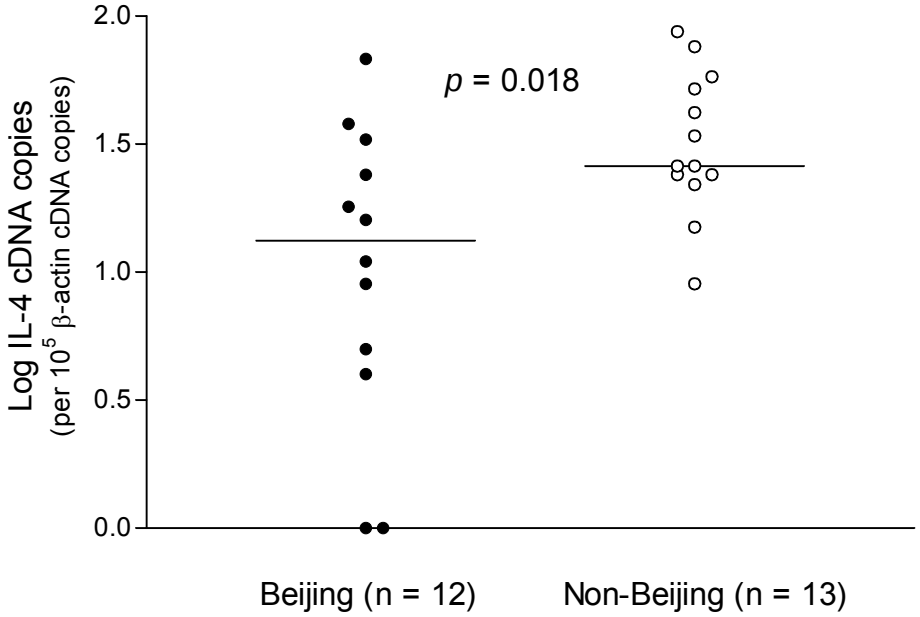

Fig. 6.10. cDNA copies of IL-4. Patients infected with non-Beijing strains had significantly higher expressed cDNA copies of IL-4 than those infected with Beijing strains ($p = 0.018$). Log medians of cDNA copies are indicated by bars.

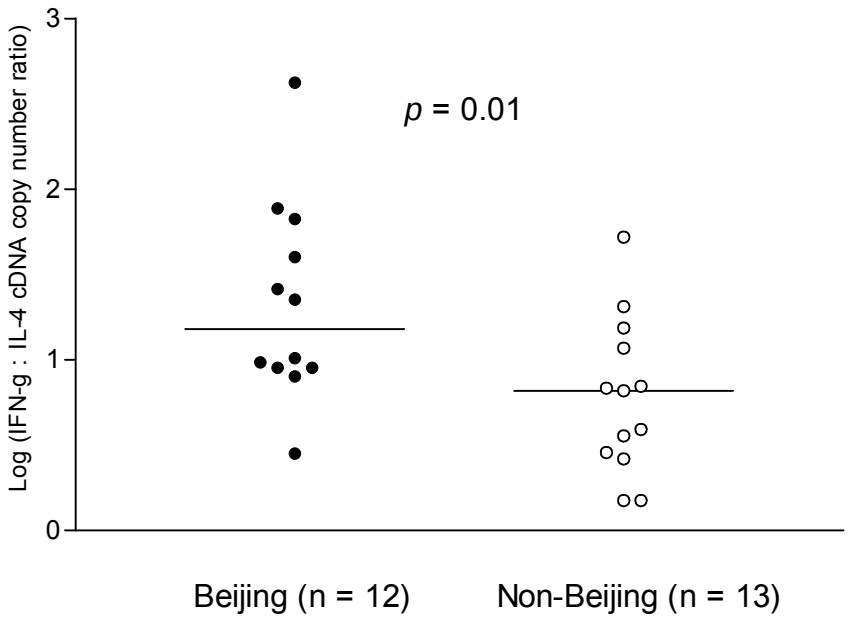

Fig. 6.11. IFN-γ : IL-4 cDNA copy number ratio of pulmonary tuberculosis patients. Patients infected with non-Beijing strains had significantly lower ratios than those infected with Beijing strains ($p = 0.01$). The bars indicate log median ratios.

We also compared the IFN-γ : IL-4 cDNA copy number ratio between the Beijing and non-Beijing groups. Two patients who had no detectable IL-4 cDNA copies were assumed having 1 cDNA copy in order to calculate the ratios. Patients infected with non-Beijing strains showed significantly lower IFN-γ : IL-4 cDNA copy number ratios than those infected with Beijing strains ($p = 0.01$) (Fig. 6.11).

6.3.7 Association between cytokines and fever

The plasma cytokine level of patients was compared by febrile and afebrile disease (Fig. 6.12). IFN-γ was significantly higher in patients with fever than in those without fever ($p = 0.004$); in addition, significantly higher of TGF-β1 was also observed in patients with fever ($p = 0.048$). There was no significant difference was found for IL-6 and IL-18

between febrile and afebrile patients. The mean plasma levels of the cytokines in febrile and afebrile patients are shown in Table 6.3.

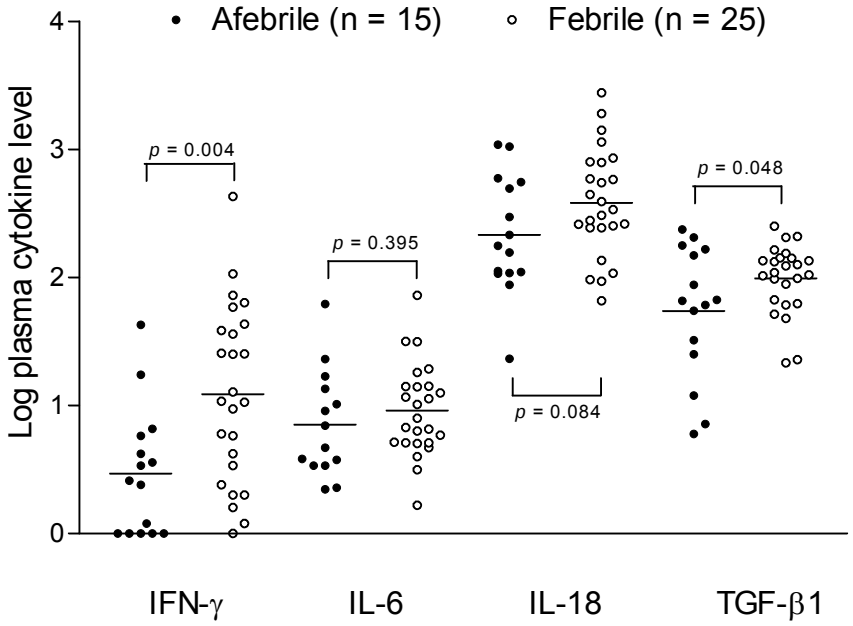

Fig. 6.12. Plasma cytokine levels of pulmonary tuberculosis patients with and without fever. Plasma level of IFN-γ, IL-6, IL-18, and TGF-β1 in pulmonary tuberculosis patients was compared by febrile and afebrile condition. Patients with febrile disease had significantly higher IFN-γ (p =0.004) and TGF-β1 (p = 0.048). Bars indicate the log mean levels in each group.

Table 6.3. Plasma cytokine level of patients by febrile and afebrile disease

Cytokines	Febrile mean ± SD	Afebrile mean ± SD	p[a]
IFN-γ (pg/ml)	40.0 ± 85.9	6.0 ± 11.1	0.004
IL-6 (pg/ml)	13.2 ± 14.7	11.1 ± 15.4	0.395
IL-18 (pg/ml)	599.0 ± 638.7	346.2 ± 345.0	0.084
TGF-β1(ng/ml)	114.5 ± 57.1	90.6 ± 77.2	0.048

[a]p is calculated based on log-transformed data.

6.3.8 Association between cytokine gene expression and cavitary tuberculosis

Of the 25 patients subjected to cytokine gene expression analysis, 6 were cavitary disease, the remaining 19 were non-cavitary disease (Table 6.2). We compared the gene expression of IFN-γ and IL-4 in patients with cavitary versus non-cavitary disease. As shown in Fig. 6.13, the cDNA copy of IFN-γ was significantly lower in patients with cavitary tuberculosis compared to those with non-cavitary tuberculosis (p = 0.028). On the contrary, the cDNA copy of IL-4 was significantly higher in cavitary disease than non-cavitary disease (p = 0.022).

The log IFN-γ : IL-4 cDNA copy number ratio of patients with cavitary disease versus non-cavitary disease is shown in Fig. 6.14. We performed logistic regression analysis to determine whether there was correlation between the ratio and cavitary disease. Interestingly, IFN-γ : IL-4 cDNA copy number ratio was significantly inversely correlated with cavitary disease [OR (95%CI), 0.551 (0.321-0.945), p = 0.0002]. The higher the ratio, the less the odds of having cavitary disease. For every one digit number increase in the ratio, the odds of having cavitary disease decreased by about 55%.

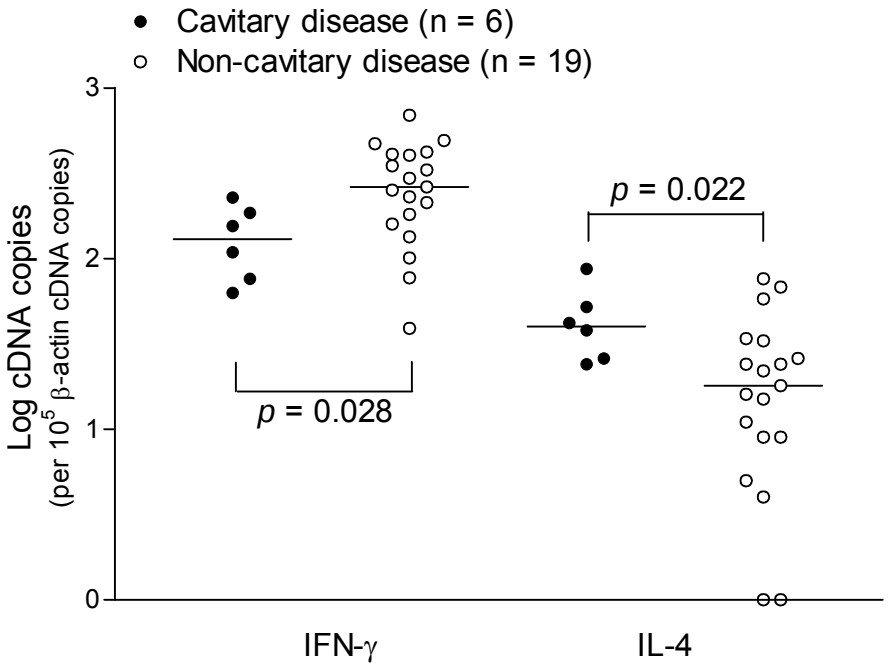

Fig. 6.13. cDNA copy of IFN-γ and IL-4 in cavitary versus non-cavitary tuberculosis. The cDNA copy of IFN-γ was significantly lower in patients with cavitary disease than those with non-cavitary disease (p = 0.028). Conversely, the cDNA copy of IL-4 was significantly higher in patients with cavitary disease than those with non-cavitary disease (p = 0.022). Log medians of cDNA copies are indicated by bars.

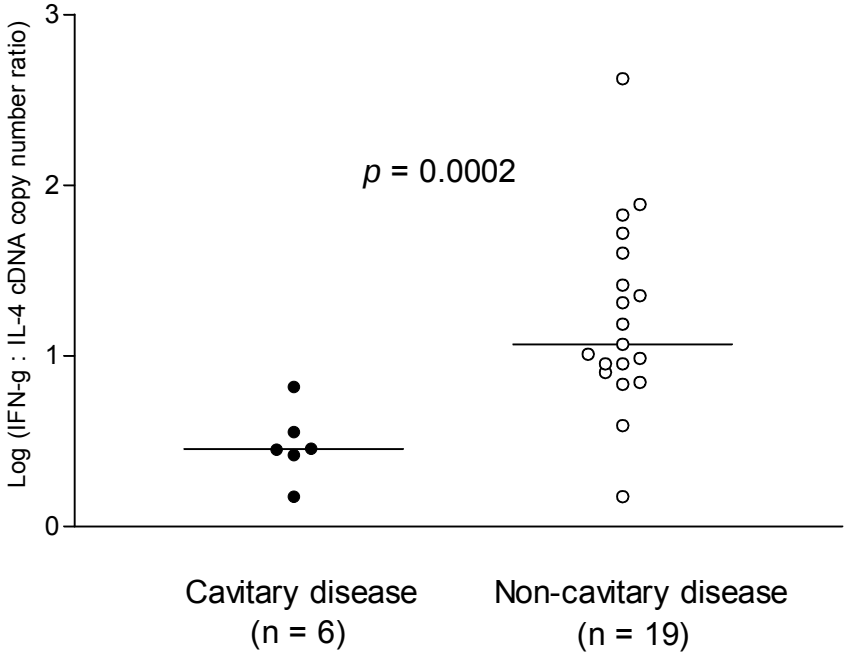

Fig. 6.14. IFN-γ : IL-4 cDNA copy number ratio of patients with cavitary versus non-cavitary tuberculosis. Cavitary tuberculosis had significantly lower IFN-γ : IL-4 cDNA copy number ratio than non-cavitary tuberculosis (p = 0.0002). The bars indicate log median ratios.

6.4 Discussion

6.4.1 Patients and clinical characteristics

In this study we recruited consecutive patients with clinically defined pulmonary tuberculosis to analyze the clinical and laboratory differences between *M. tuberculosis* genotypes. All the subjects had microbiologically confirmed tuberculosis, and the manner of recruitment and pattern of symptoms suggests that they are likely to be representative of the population of tuberculosis patients presenting to a typical general practitioner, polyclinic or hospital emergency department in Singapore. In spite of the reluctance of some patients to undergo HIV testing, we were able to definitively exclude HIV co-infection in many of the participants, and establish the absence of risk factors in the remainder. Given that the rate of HIV coinfection in tuberculosis patients is less than 1% in Singapore (unpublished data), it is unlikely that a significant proportion of the remainder would be HIV positive. Although the study population was half the size estimated to be necessary to address the primary hypothesis of the study (namely that there is a difference in IFN-γ between Beijing and non-Beijing strains), we were nonetheless able to make some useful clinical observations that may form the basis of future clinical studies.

Fever is a common manifestation of pulmonary tuberculosis and is one of the few that can be measured objectively. The frequency with which fever has been observed in patients with tuberculosis varies widely from approximately 34% to 94% (Arango et al., 1973; Kiblawi et al., 1981; Rathman et al., 2003). Patients with fever are more often symptomatic, more likely to have advanced disease, more likely to be smear positive, and tend to be younger (Kiblawi et al., 1981; Barnes et al., 1987). In this study we made the

interesting and potentially very important observation that 85% of patients infected with non-Beijing strains had fever, whereas only 43% of those infected with Beijing strains had fever, and this large difference is statistically significant.

We used an objective definition of fever that depended on objective observations made while patients were hospitalized, and taken by staff who were obviously unaware of the genotype of *M. tuberculosis* with which the patient is infected. In addition to fever, we also found that night sweats were significantly less common in patients infected with Beijing strains. This latter finding agrees with the findings of a large study performed in Russia by Drobniewski et al. (Drobniewski et al., 2005) to examine differences between patients infected with Beijing and non-Beijing strains. In that study, night sweats were found significantly less often (OR, 0.7) in patients infected with Beijing strains and the association remained significant after multivariate adjustment. However, in that study there was no significant difference in the frequency of fever between Beijing and non-Beijing groups. The definition of fever was not given and the overall rate (approximately 30% in both groups) suggests that some under-reporting of fever may have occurred. Although we found that the frequency of fever and of night sweats differed between the Beijing and non-Beijing groups, after multivariate analysis only fever remained significant. This is not surprising as the two symptoms often occur together in the same patient, and so it is difficult to ascertain an independent effect. The important point is that our study agrees with the study from Russia that there is a difference in the clinical systemic host response to Beijing and non-Beijing genotype strains.

This study has demonstrated the significantly lower frequency of fever, night sweats, and of cavitation in patients infected with Beijing genotype strains, moreover, a

number of other symptoms, such as cough, hemoptysis, and chest pain, were also less common, although not significant, in tuberculosis caused by Beijing strains. This suggests that tuberculosis caused by Beijing strains is less symptomatic and less severe compared to disease caused by non-Beijing strains. Symptom, particularly fever, is important for patients to recognize that they have a condition that may need medical attention, and hence causes them to seek medical attention. The absence of symptom(s) and/or less severe disease may therefore lead to delays in consultation and/or make patients less identifiable, thus increasing the likelihood of transmission in the case of tuberculosis. To examine this hypothesis, we compared the duration of cough of the subjects before the diagnosis by Beijing and non-Beijing genotypes, because cough is the most common way of spreading the bacteria. We found that patients infected with Beijing strains had a significantly longer duration of cough compared to those infected with non-Beijing strains. This may partially account for why the *M. tuberculosis* Beijing genotype is more prevalent. The data of cough duration was based on the patients' subjective description, and this may not be very accurate. Furthermore, other socioeconomic factors may influence patient's decision for consultation. Therefore, a larger, well-designed study is needed to answer questions about the consequences of the differences in symptoms on presentation, diagnosis, and transmission of tuberculosis.

The *M. tuberculosis* Beijing genotype has been recently identified as a bacterial factor that is associated with febrile response to treatment in Indonesia (van Crevel et al., 2001). In the present study, we did not collect the temperature data after the initiation of therapy because a high proportion of patients were discharged upon commencing of treatment, and hence we are unable to compare our results directly with theirs. In

addition, van Crevel et al. (van Crevel et al., 2001) do not elaborate on the presence of fever in their subjects prior to treatment, which would have provided useful comparative data. It is certainly possible that the pre-treatment and post-treatment inflammatory clinical response may differ between *M. tuberculosis* genotypes, and this represents an interesting area for further study.

We found that there was a significant higher frequency of cavitary disease in patients infected with non-Beijing strains (OR, 0.2). However, in studies performed in Indonesia (van Crevel et al., 2001) and in The Netherlands (Borgdorff et al. 2004) in which the CXR presentations of patients infected with Beijing and non-Beijing strains were compared, the authors did not find any significant difference between the two groups of patients. But in the much larger study by Drobniewski et al. (2005), infection with Beijing genotype was associated more commonly with advanced radiological abnormalities, defined as multiple lung zones with fibrotic change and widespread cavitation, than was non-Beijing genotype, a result that is somewhat contrary to ours.

In contrast to our observation in the present study that patients infected with Beijing strains presented with less severe disease, two previous studies (Manca et al., 2001; López et al., 2003) found that Beijing strains caused more severe disease in mice, as demonstrated by higher earlier mortality of infected mice. The hypervirulence of the Beijing strains in the mouse studies might be due to the way that the disease was presented as a challenge, and that the immunity was unable to arrest the infection; especially in the study by López et al. (2003), the mice died before or soon after the development of an adaptive immunity which needs 3 weeks to develop in mice and actually innate immunity appears to be not protective in mice (North and Jung, 2004).

But in patients, active disease is usually a result of reactivation of latent infection in which an adaptive immunity is dominant.

6.4.2 Laboratory parameters

We found that patients infected with non-Beijing strains had significantly higher levels of peripheral blood monocytes. This proliferation of monocytes in the non-Beijing group might be related to higher frequency of febrile disease as the products of monocytes IL-1, IL-6, and TNF-α are potent endogenous pyrogens (Netea et al., 2000a). Monocytes are key regulators of immune responses, essential for the generation of beneficial antipathogen immune reactions. Peripheral blood monocytes are recruited, by signals such as chemokines, from the circulation to the sites of inflammation caused by *M. tuberculosis* infection. Upon activation at the sites of inflammation, monocytes can modulate the immune reactions by producing a number of cytokines, such as IL-1, IL-6, IL-10, IL-12, TNF-α, and TGF-β (Wallis et al., 1990; Barnes et al., 1993; Toossi et al., 1995; Fulton et al., 1996). IL-1, IL-6, IL-12, and TNF-α are proinflammatory cytokines; IL-12 is a central player in induction of IFN-γ, favoring the development of Th1 protective immunity (van Crevel et al., 2002); TNF-α can exhibit both protective and pathological effects depending on its production; physiological concentrations of TNF-α play a key role in granuloma formation but excessive production of TNF-α may cause marked tissue necrosis that is characteristic of progressive disease (Barnes and Modlin, 1996); IL-10 and TGF-β are anti-inflammatory cytokines; they can antagonize the proinflammatory cytokine responses, such as suppression of production of IFN-γ and TNF-α by down-regulation. In addition, differential proliferation of human T cell subsets

in response to *in vitro* stimulations of mycobacteria or mycobacterial antigens has been observed in the presence or absence of monocytes (Esin et al., 1996).

6.4.3 Cytokine response to infection of Beijing and non-Beijing strains

This study has demonstrated that plasma levels of IFN-γ did not differ significantly between patients infected with Beijing and non-Beijing strains. Thus the primary hypothesis of this study, namely that IFN-γ production would be lower in pulmonary tuberculosis patients infected with Beijing strains compared with those infected with non-Beijing strains was not confirmed. However, although the study represents a substantial body of work, it is important to note that recruitment was terminated early (for the reasons noted in section 6.3.1) after only approximately half the estimated necessary numbers of subjects had been recruited. The group mean and standard deviation of the IFN-γ results was similar to that used in the power calculation, and hence the study may be underpowered to detecte a difference between groups of the magnitude that was originally estimated (100%). Thus, although there appeared to be no major difference in IFN-γ between Beijing and non-Beijing groups in this study, it is possible that this represents a Type II error.

We also measured gene expression level for a number of cytokines. The absence of a difference in plasma IFN-γ levels is corroborated by the absence of a difference between the two groups of patients in IFN-γ gene expression level in PBMC. Moreover, the plasma level and gene expression level of IL-18, an important inducer for IFN-γ and maybe also for other proinflammatory cytokines (Dinarello et al., 1998), were also not different between the two groups of patients. Although we could not detect IL-2 in

plasma, its gene expression in PBMC was not different in the Beijing and non-Beijing groups. These findings in general do not suggest that Beijing strains elicit a weaker Th1 immune response in tuberculosis patients, and differ from the observation from mouse studies that mice infected with the Beijing strains presented a reduced Th1 immunity (Manca et al., 2001; López et al., 2003).

However, we found that patients infected with non-Beijing strains had significant higher level of gene expression of IL-4 in PBMC and significant lower IFN-γ : IL-4 cDNA copy number ratio compared to those with Beijing strains, suggesting an enhanced Th2 immune response in patients infected with non-Beijing strains. This finding to some extent agrees with a recent *in vitro* study (Chacón-Salinas et al., 2005), in which macrophages derived from bone marrow of mice were infected with different *M tuberculosis* genotype strains. They found that cells infected with Beijing strains induced a Th1 favorable immune response as evidenced by the highest mRNA and protein expression of IL-1β, TNF-α, and IL-12, and a comparable IL-18 with other *M. tuberculosis* genotype strains, and on the other hand, a reduced Th2 immune response in cells infected with Beijing strains as demonstrated by diminished IL-10 mRNA compared with other non-Beijing strains. This cytokine pattern is favorable to control infection.

In an *in vitro* study (Manca et al., 2004) in which PBMC from healthy donors were infected with an outbreak Beijing strain HN878 and other Beijing strains as well as non-Beijing strains, the infections of the Beijing strains were characterized by Th2-polarized immunity with preferentially induced IL-4 and IL-13. This differs from the findings in the present study, and the reason that was responsible for the difference needs to be elucidated.

Many (IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, and TNF-α) of the cytokines were not detected in the unstimulated blood of tuberculosis patients by conventional ELISA assays in the present study. Some of these negative observations have been frequently reported in a number of *in vivo* studies with human subjects (Barnes et al., 1993b; Zhang et al., 1994; Verbon et al., 1999; Olobo et al., 2001; Vankayalapati et al., 2003), though inconsistent results are also observed among these studies. The reasons responsible for the disparities in blood cytokine concentrations among studies are multiple and complicated. Variations in individual patient, sampling timing, sample processing (serum or plasma), assay sensitivity, and experimental personel error, *etc*. may account for some of the differences. But some constitutional factors as discussed below are likely the primary reasons, at least for some of the cytokines.

Firstly, cytokine genes are expressed at very low level. This is seen in the results of IL-2, IL-4, and IL-13 in this study. These undetectable cytokines by ELISA were all at very low gene expression levels (Figs. 6.8 and 6.10); moreover, IL-13 gene expression was not even detected by the real-time quantitative cDNA PCR. This is consistent with previous studies (Barnes et al., 1993a; Schauf et al., 1993). Therefore, these cytokines were either undetectable (Barnes et al., 1993b; Swaminathan et al., 1999) or at very low concentrations in unstimulated blood (Verbon et al., 1999; Dheda et al., 2005). With the exception of IL-4, it is not known whether these cytokines are biologically active at undetectable low concentration except IL-4. IL-4 is thought to be biologically active at very low concentration (reviewed by Brown and Hural, 1997 and Rook et al., 2005b). In addition, IL-4 appears to be present at significant different concentrations between people in developed and developing countries because it is consistently not detectable in

tuberculosis patients from developed countries but easily detected in those from developing countries (reviewed by Rook et al., 2005b). Although IL-5 gene expression was not measured in the present study, a previous study (Barnes et al., 1993b) has shown that both IL-5 protein and mRNA were not detectable *in vivo*, suggesting that IL-5 is likely a member of the cytokines that express *in vivo* at very low level.

Secondly, cytokines are mainly produced at the local site of disease, particularly in the case of IL-12 and TNF-α. IL-12 is a heterodimeric 70-kDa protein composed of two subunits (35 kDa light chain, p35 and 40 kDa heavy chain, p40) linked by a disulfide bond (Kobayashi et al., 1989; Stern et al., 1990). Although IL-12 is thought to be a major player in the protective immunity against tuberculosis, this concept is mainly based on evidence from *in vitro* stimulation studies with human cells, from individuals with IL-12 receptor gene defects, and from gene knock-out mouse studies (reviewed by van Crevel et al., 2002). In keeping with the findings of the present study, the biologically active IL-12p70 has never been detected from the serum or plasma of tuberculosis patients in previous studies (Zhang et al., 1994; Verbon et al., 1999) although it has been demonstrated to be present in tuberculous pleural fluid (Zhang et al., 1994). However, the IL-12p40 subunit is easily detected from the blood of tuberculosis patients (Zhang et al., 1994; Verbon et al., 1999). IL-12p40, however, does not essentially reflect the presence of IL-12p70 because this subunit is shared by another different interleukin, known as IL-23, which is an inducer of Th17 cells and cytokine (IL-17) (reviewed by Langrish et al., 2004). These findings suggest that IL-12 may be only produced at the site of disease and hardly spread into the circulating system. In addition, the absence of circulating IL-12p70 from tuberculosis patients may imply the presence of a novel pathogenic mechanism for

tuberculosis because it has been found that the absence of IL-12p70 can promot an increase in the number of IL-17-producing Ag-specific CD4 T cells both *in vitro* and *in vivo* (Khader et al., 2005) while IL-17 is a mediator of inflammatory responses and increased levels of IL-17 has been associated with several inflammatory conditions and tissue damage (reviewed by Witowski et al., 2004).

Although TNF-α is present at much higher concentration in local fluids (pleural fluid and bronchoalveolar lavage fluid) of disease sites than in blood (Barnes et al., 1990; Tsao et al., 1999a, 1999b; Gursel et al., 1995; Olobo et al., 2001), there have been many reported studies from South Korea (Kim et al., 1991), Turkey (Gursel et al., 1995; Cagatay yet al., 2005), South Africa (Bekker et al., 1998), Argentina (Dlugovitzky et al., 1999; Fiorenza et al., 2005), Spain (Poveda et al., 1999), Taiwan (Tsao et al., 1999a, 1999b), and Brazil (Ribeiro-Rodrigues et al., 2002), in which TNF-α is detected in either serum or plasma from tuberculosis patients. However, other studies, for example from the Netherlands (Juffermans et al., 1998) and Ethiopia (Olobo et al., 2001) that have found TNF-α to be undetectable in the sera of tuberculosis patients. The South Korean study (Kim et al., 1991) has shown that TNF-α concentration in lungs seems an important factor to affect TNF-α concentration in bloodstream; no serum TNF-α was detected if TNF-α concentration in lungs is low. We did not measure the cytokines in lungs, but a low level expression of TNF-α is likely a reason why it is not detected in our patients.

IL-10 is a potent anti-inflammatory and immunosuppressive cytokine which can inhibit the production of many cytokines, such as IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-12, IL-18, etc. (reviewed by Moore et al., 2001). Like IL-12 and TNF-α, IL-10 appears to be

produced mainly at the site of disease (Olobo et al., 2001). It has been shown that serum IL-10 is elevated in tuberculosis patients compared to healthy controls (Dlugovitzky et al., 1997; Verbon et al., 1999; Morosini et al., 2003; Vankayalapati et al., 2003). However, in the studies performed by Verbon et al. (1999) and by Olobo et al. (2001) in which IL-10 measurement of each individual patient is presented, serum IL-10 was detected in only about 17% (14/81) and 42% (10/24) of the tuberculosis patients, respectively; moreover, serum IL-10 was also elevated in the contacts and healthy controls in the study by Olobo et al. (2001) but not in the study by Verbon et al. (1999). Therefore, the elevated serum IL-10 in tuberculosis patients demonstrated in these studies does not essentially mean that it was due to tuberculosis. In addition, because serum IL-10 level does not parallel *M. tuberculosis*-induced IL-10 production by PBMC *in vitro* in the same patients, it has been speculated that the IL-10 in bloodstream is due to leakage of IL-10 from tissue into the circulation (Vankayalapati et al., 2003). Therefore, possibly similarly as TNF-α, a low level expression of IL-10 is likely a reason why it is not detected in our patients.

Although it is possible to conduct *ex vivo* stimulation of cells to increase cytokine production, this may not yield an accurate reflection of the cytokine balance present *in vivo* (Rook et al., 2001). Indeed, a recent study has shown that the concentrations of IFN-γ, IL-18, and IL-10 in serum do not parallel cytokine levels produced by *in vitro M. tuberculosis*-stimulated PBMCs regardless of they are from tuberculosis patients or from healthy tuberculin reactors (Vankayalapati et al., 2003). However, a major shortcoming in our cytokine experiments is that we did not set up healthy subject controls.

6.4.4 Relationship between clinical parameters and cytokines

We have shown that pulmonary tuberculosis patients with fever had higher plasma IFN-γ and TGF-β1 compared to those without fever. This confirmed the previous finding that active tuberculosis patients with fever showed higher serum IFN-γ than those without fever (Verbon et al., 1999). But we did not find a significant higher level of IL-6 in the patients with fever, a result that is not consistent with the Verbon study (Verbon et al., 1999). This may be due to the small sample size. Fever is a systemic biological response to exogenous pyrogens mediated by endogenous pyrogens, namely proinflammatory cytokines including IL-1, IL-6, TNF-α and interferons (Netea et al., 2000a). IL-1, IL-6, and TNF-α are potent endogenous pyrogens; IL-6 seems at the critical position in the cascade of cytokines acting as endogenous pyrogens in the pathogenesis of fever, IL-1 and TNF-α function as pyrogen through IL-6. IFN-γ may be pyrogenic through induction of IL-1 and TNF (Netea et al., 2000a). In the present study, IFN-γ, IL-6, IL-18, and TGF-β1 were all higher in tuberculosis patients with fever, suggesting stronger inflammatory responses in these patients.

IFN-γ gene expression level was significantly higher in non-cavitary tuberculosis than was in cavitary tuberculosis. Conversely, IL-4 gene expression level was significantly lower in non-cavitary disease than was in cavitary disease. More interestingly, the IFN-γ : IL-4 cDNA copy number ratio was significantly correlated with non-cavitary disease. Our findings agree well with previous studies (Seah et al., 2000; van Crevel et al., 2000; Mazzarella et al., 2003; Bai et al., 2004). In the *in vivo* study by Seah et al. (2000), both higher IL-4 gene expression in PBMC and lower IFN-γ : IL-4 cDNA copy number ratio were found to be related with more extensive radiographic

abnormality and with pulmonary cavities. In another *in vivo* study by Mazzarella et al. (2003), a predominant Th1 cellular immune response was observed in the lungs of non-cavitary tuberculosis patients, while cavitary involved lung segments exhibit the presence of Th2 lymphocyte subset. In the study by van Crevel et al. (2000), *in vitro* stimulated CD4+ and CD8+ T cells from cavitary tuberculosis patients produced higher IL-4 than those from non-cavitary tuberculosis patients. Bai et al. (2004) have recently reported that IL-4 and IL-13 decreased in well-formed pulmonary granulomas of tuberculosis patients, suggesting that the absence of IL-4 and IL-13 is essential for granuloma formation. In addition, in BALB/c mouse model of tuberculosis, progressive disease and reactivation of latent infection (Hernandez-Pando et al., 1996a, 1996b) are both associated with increased production of IL-4. In IL-4 gene knockout (KO) BALB/c mice, IL-4 was found to have modestly detrimental effects on the antibacterial efficacy of the Th1 response, and larger effects on the toxicity of TNF-α and on fibrosis (Hernadez-Pando et al., 2004). Moreover, overexpression of IL-4 intensified tissue damage in experimental infection (Lukacs et al., 1997).

Taken together, our observations that IL-4 expression was significantly elevated in patients infected with non-Beijing strains but not in those infected with Beijing strains and these non-Beijing patients had more febrile and cavitary disease strongly suggest that IL-4 plays a pathogenic role in tuberculosis, especially in development of cavitary disease. Hence, although there are some KO mouse studies do not support a pathogenic role for IL-4 in tuberculosis disease (North, 1998; Jung et al., 2002), Rook et al. (2004) have argued that overproduction of IL-4 is a cause instead of an effect in tuberculosis and thought that IL-4 is a pathogenic factor for fibrosis in lung.

The significant inverse correlation between IFN-γ : IL-4 cDNA copy number ratio and cavitary tuberculosis suggests that Th1 and Th2 immunity must maintain some extent of balance. If the balance is impaired, active disease would be developed from latent infection (Ordway et al., 2004) or cavitary disease would be developed (van Crevel et al., 2000, and this study). In this study, all the cavitary tuberculosis had an IFN-γ : IL-4 cDNA copy number ratio less than 10, and the low ratio was not only due to increased IL-4 (Th2 immunity) gene expression but also due to reduced IFN-γ (Th1 immunity) gene expression. This further suggests the pathogenic role of IL-4 because the reduced IFN-γ gene expression was likely a result of down regulation by over expressed IL-4.

6.4.5 Conclusions

This study suggests that the *M. tuberculosis* Beijing genotype strains may clinically not be as virulent as previously expected in tuberculosis patients; the less symptomatic and cavitary disease caused by Beijing strains suggests that they may be clinically less virulent. Importantly, the less symptomatic and/or severe disease associated with Beijing strains may result in delay for consultation and/or clinical overlook in the identification of patient, thus increasing the risk of transmission. This could be a reason accounting for the high prevalence and more involvement of Beijing strains in transmission.

Th1 immune response is comparable in patients infected with *M. tuberculosis* Beijing versus non-Beijing strains. A Th2-polarized immunity was observed in patients infected with non-Beijing strains, though in contrast with previous *in vitro* findings (Manca et al., 2004). This observation, corroborating with previous findings in human tuberculosis studies (Seah et al., 2000; van Crevel et al., 2000; Mazzarella et al., 2003;

Bai et al., 2004), can be a rational immunological basis with which to interpret the clinical features of our subjects. Taken together, all the findings from our study and from the others indicate the intriguing complexity of tuberculosis immunity; it may vary markedly in different hosts. This has implications for interpreting results from different hosts and the importance of *in vivo* study in tuberculosis patients as the final determinant.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS OF RESEARCH

7.1 Genotyping of *M. tuberculosis*

Key factors in the control of tuberculosis are rapid detection, adequate therapy, and contact tracing to arrest further transmission. Genotyping of *M. tuberculosis* isolates is useful molecular epidemiological means to study transmission and to trace epidemiologically-related cases. As such, typing all *M. tuberculosis* isolates using IS*6110* RFLP typing has been performed routinely in many developed countries, such as The Netherlands, Denmark, and Norway. In the USA, more than 25 states perform this typing. In other countries, such as Germany, Italy, France and Spain, routine typing is mostly reserved for resistant isolates (van Soolingen et al., 2003). An international IS*6110* RFLP database has been set up in The Netherlands which has greatly facilitated tuberculosis studies conducted either in that country (van Soolingen et al., 1999; van Deuketom et al., 2004, 2005) or abroad (Suffys et al., 2000; Siddiqi et al., 2001). Due to resource limitations, routine typing of *M. tuberculosis* isolates in the developing world remains impossible in spite of the fact that the vast majority of tuberculosis cases are in developing countries. In Singapore, it has been suggested that strain-typing should be adopted as a molecular epidemiological measure to enhance the control of tuberculosis (Chee and James, 2003).

Since its advent, it has been suggested that MIRU-VNTR typing could be applied directly on clinical specimens (Mazars et al., 2001; Supply et al., 2001), like spoligotyping. However, such application has not been reported until this study. The automated, high throughput format of MIRU-VNTR typing based on a genetic analyzer to detect fluorescent dye labeled PCR products has shown excellent sensitivity using either slab gel electrophoresis (Supply et al., 2001) or capillary electrophoresis (Sun et

al., 2004a) because of the employment of sensitive fluorescence detection technology. Indeed, in this study, using only 2 μl of total DNA samples extracted directly from sputum in each PCR reaction, we were able to amplify the MIRU-VNTR locus sequences and successfully typed 41 of 42 culture-proven tuberculosis isolates. This indicates the great potential of MIRU-VNTR typing to be used as the first line, "real-time" *M. tuberculosis* genotyping method, or maybe as a diagnostic method.

Our work has demonstrated the feasibility of typing *M. tuberculosis* isolates as a routine using MIRU-VNTR typing as the first-line modality and reserving IS*6110* RFLP typing as a secondary modality to subdivide isolates that are clustered in MIRU-VNTR typing. With direct application to clinical specimens, such as sputum, labor-intensive subculturing (for IS*6110* RFLP) will only be required for selected isolates that are clustered by MIRU-VNTR typing. This will greatly reduce the workloads of subculturing isolates and provide "real-time" typing results for most of patients. Furthermore, MIRU-VNTR typing may be also used as a rapid detection method.

7.2 Prevalence of Beijing genotype over time in Singapore

It has been reported that the Beijing genotype of *M. tuberculosis* is emerging in Vietnam (Anh et al., 2000), in Russia (Drobniewski et al., 2005), and in The Netherlands (Glynn et al., 2005). Therefore, it is of local and global interest to confirm the emerging trend of this genotype because it has important implications for the control or even vaccine design of tuberculosis. Comparison of the genotyping results of our two sets of samples that were separated by an interval of 10 years, suggests that there has been no major change in the prevalence of the Beijing genotype. In 1994, Beijing strains accounted for

approximately 54% (196/364, see Chapter 2) of new drug-susceptible tuberculosis, and 10 years later it was approximately 59% (17/29, see Chapter 6) in local residents. The proportions of Beijing strains in the two samples were not different ($p = 0.43$). However, the two sampling populations were different in that the isolates collected in 1994 (Chapters 2) represented the then population of drug-susceptible *M. tuberculosis*, whereas the samples collected in 2004 and 2005 (Chapter 6) represented patients with clinically evident (and predominantly smear positive) tuberculosis who were hospitalized for investigation and initiation of treatment. Thus this comparison needed to be interpreted with caution.

7.3 *M. tuberculosis* Beijing genotype: New perspectives

One salient finding in this project is that Beijing strains appeared to cause less symptomatic disease compared to non-Beijing strains. Possibly because of this, patients infected with Beijing strains took longer to be identified, which may increase the likelihood of spreading the bacilli. This might be a 'selective advantage' associated with the highly prevalence of *M. tuberculosis* Beijing strains. Due to the weaknesses of our study as discussed in Chapter 6, a larger, well-designed molecular epidemiological study is needed to confirm this finding. In such a study the correlation of the time between disease onset and being diagnosed with transmission clusters defined by *M. tuberculosis* DNA fingerprints should be investigated on the basis of excluding the effects from demographic and socioeconomic factors of study subjects that may affect patient's will to seek medical attention.

One hypothesis is that the Beijing genotype is an 'escape variant' from the immune protection provided by BCG vaccination, so BCG vaccination can provide protection to other genotype strains but not against this 'Beijing variant' (van Soolingen et al., 1995). Because of the very high coverage of BCG vaccination in areas with high prevalence of Beijing strains, no association between BCG vaccination and Beijing strains has been established until recently. A recent multicentre study, involved in The Netherlands, Vietnam, and Hong Kong, has demonstrated an association, albeit rather weak, between BCG vaccination and Beijing genotype strains (Kremer et al., 2005). However, based on our observations, we postulate that the *M. tuberculosis* Beijing genotype may be an "attenuated variant" resulted from the interaction between its progenitor and the immune defense of BCG vaccination. Alternatively, BCG vaccination may provide some extent of protection against the virulence of Beijing strains, thus they cause less symptomatic and less severe disease. In view of the high virulence of Beijing strains in mice (Manca et al., 2001; López et al., 2003) and in *in vitro* cultured cells (Manca et al., 2004), as well as their rapid spreading in areas without or with low BCG coverage (Caminero et al., 2001; Glynn et al., 2005), the latter is perhaps more plausible. Future studies on this aspect would be interesting.

7.4 The pathogenic role of IL-4 in tuberculosis

As an anti-inflammatory cytokine, IL-4 is deleterious in intracellular infection, such as suppression of Th1 response (Biedermann et al., 2001), suppression of macrophage activation (van Crevel et al., 2002), switch of signaling via TLR-2 and down regulation of iNOS (Bogdan et al., 1994). In human tuberculosis, it has been found that IL-4 can

suppress the production of IL-2 and is predominant in active disease (Lucey et al., 1996). Studies have also demonstrated that increased IL-4 production is associated with advanced radiological disease (Seah et al., 2000), with cavitary disease (van Crevel et al., 2000), and with progression from latent infection to active disease (Ordway et al., 2004). All these data suggest a pathogenic role for IL-4 in tuberculosis. However, evidence from mouse studies are contradictory. In BALB/c mice infected with *M. tuberculosis*, progressive disease and reactivation of latent infection are both associated with increased production of IL-4 (Hernandez Pando et al., 1996a, 1996b), and overexpression of IL-4 intensified tissue damage in experimental infection (Lukacs et al., 1997). These are in agreement with the findings from humans. On the other hand, results from studies with KO mice do not support a pathogenic role for IL-4 in mouse tuberculosis (North, 1998; Jung et al., 2002). Our study represented the first *in vivo* human study that has demonstrated the different IL-4 responses in patients infected with Beijing and non-Beijing strains. In the patients infected with non-Beijing strains, IL-4 expressed markedly higher and that was correlated with severe disease, such as fever and cavitation. These linkages among etiological pathogen, disease development mediator, and disease presentation strongly suggest that IL-4 is a pathogenic factor in tuberculosis. The establishment of IL-4 as a pathogenic cytokine in tuberculosis has important implications for therapy and for vaccine design. Studies on this direction are essential to shed more light on issues, such as in what way *M. tuberculosis* overactivates IL-4 expression, and how IL-4 causes disease.

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APPENDICE
Appendix 1

Figure 2.1. Spoligotyping patterns of the 364 drug-susceptible isolates. A total of 118 distinct spoligotyping patterns were generated. The patterns were grouped by the families of *M. tuberculosis* based on the common characteristics of spoligotypes of each family. The S family is a novel clone defined in this study (see Chapter 3).

Families	Pattern No.	Spoligotyping patterns	No. of isolates
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	3	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■□■■■	1
	4	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■□■	4
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	18	■■□■■■■■■■■■□■■■■■■□□■■■■■■■□□□□■□■■■■■■■■■	1
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	20	■■□■■■■■■■■■■■■■■■■□□■■■■■■■□□□□■□■■■■■□■■■	1
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	28	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■■■■■	1
	29	■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■□■■■■■□■■■	1
	30	■■□■■■■■■■■■■■■■■■■■■■■■□□■■□□□□■□■■■■■□■■■	1

Families	Pattern No.	Spoligotyping patterns	No. of isolates
EAI			
	31	■■■■■■■■■■■■■■■■■■■■■■■□■□□■■□□□□■□■■■■■■■■■	1
	32	■■■■□■■■■■■■■■■■■■■■□■■■■■□□□□■□■■□■■■■■■	1
	33	■■□■■■■■■■■■■■■■■■■■■■□■■■■■□□□□■□■■■■■■■■■	1
	34	■■■■■■■■■■■■■■■□□■■■■■■■■□■■□□□□■□■■■■□■■■■	1
	35	■■■□□■■■■■■■■■■■■■■■■■■■□■■□□□□■□■□■■■□■■■	1
	36	■□□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□■■■■	10
	37	■□□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□□■■■	1
	38	■□□■■□■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□■■■■	2
	39	■□□■■■■■■■■■■■■■■■■■■■□■■■■□□□□■□■■□□□■■■■	1
	40	■□□■■■■■□■■■■■■■■■■■■■■■■■□□□□■□■■□□□□■■■	1
	41	■■■■■■■■■■■■■■■■■□■■■■■■■■□□□□■□■■□□□■■■■	1
	42	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■□■■■■	1
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	59	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□■□□□□■■■■■■■	1
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Families	Pattern No.	Spoligotyping patterns	No. of isolates
Haarlem			
	71	■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□■□□□□■■■■■■■	1
	72	■■■■■■■■■■■□■■■■■■■■■■■■□□□□□□□■□□□□■■■□■■■	1
	73	■■■■■■■■■■■■■□■■■■■■■■□□□□□□□■□□□□■■■■■□■	1
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	88	■■	2
	89	■■■□■■	1
	90	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■	1
	91	■■■□■■■	1
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	94	■■■□□□■■■■■■■■■■■■□□□■■■■■■■■■■■■■■■■■■■■■	1
	95	■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□□□	1
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	98	■■■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■□□□□■■■■■■■	1
	99	■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1
	100	■■■■■■■■■■■■□■■■■■■■□■■■■■■■■■■□□□□■■■■■■■	1
	101	■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1
	102	■■■□■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1
	103	■■■■■■■■■■■■□■□□■■■■■■■■■■■■■■■■□□□□■■■■■■■	1
	104	■■■■■■■■■□■■■■□■■■■■■■■■■■■■■■■□□□□■■□□■■■	1
	105	■■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	1
	106	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□□	3
	107	■■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□□■■	1
	108	■■■■□■■■■□■■■■■■■■■■□□■■■■■■■■■□□□□■■■□■■■	1
	109	■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■□□□□■■■■■■■	1
	110	■□□□□■■■■■■■□■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	1

Families	Pattern No.	Spoliotyping patterns	No. of isolates
T			
	111	■■■■■■■■■□■■■■□■■■■■■■■■■■□■■■■□□□□□■■□■■□■	1
	112	■□■□□□□■■■■■■■□■■■■■■■■■■■■■■■■□□□□□■□■■□■	1
	113	■■■□□□□□□□■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	2
	114	■■■■□□□□□□□□□■□■■■■■■■■■■■■■■■■□□□□■■■■■■■	2
	115	■■■■■■■■■□■■□■□□■■■■■■■□□□□□■■■■□□□□■■□■■□■	1
x			
	116	■■■■■■■■■■■■■□■■□■■■■■■■■■■■■■□□□□■■■■■■■	1
	117	□□□□□□□□□□□□□■■■■■□■■■■■■■■■■■■■□□□□■□□■■■■	1
	118	■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■□□□□■■□□□□■	1

Appendix 2

Table 2.3. MIRU-VNTR patterns of drug-susceptible isolates

Families	Pattern No.	MIRU-VNTR loci												No.of isolates
		2	4	10	16	20	23	24	26	27	31	39	40	
Beijing														
	1	2	0	3	3	2	5	1	7	3	5	2	3	1
	2	2	0	3	3	2	5	1	7	3	5	3	3	2
	3	2	2	1	3	2	5	1	7	3	5	3	3	1
	4	2	2	1	3	2	7	1	7	3	5	3	4	1
	5	2	2	2	2	2	5	1	7	3	5	4	3	1
	6	2	2	2	3	2	5	1	1	3	3	2	2	1
	7	2	2	2	3	2	5	1	5	2	3	2	2	1
	8	2	2	2	3	2	5	1	5	3	3	4	2	2
	9	2	2	2	3	2	5	1	6	3	5	4	3	2
	10	2	2	2	3	2	5	1	7	1	5	4	1	1
	11	2	2	2	3	2	5	1	7	3	3	4	3	1
	12	2	2	2	3	2	5	1	7	3	5	2	3	3
	13	2	2	2	3	2	5	1	7	3	5	3	3	10
	14	2	2	2	3	2	5	1	7	3	5	4	3	17
	15	2	2	2	3	2	5	1	9	3	5	4	3	2
	16	2	2	3	1	2	5	1	7	3	4	3	3	1
	17	2	2	3	2	2	5	1	5	1	5	3	3	2
	18	2	2	3	2	2	5	1	5	2	5	2	2	1
	19	2	2	3	2	2	5	1	7	3	5	3	3	4
	20	2	2	3	3	1	5	1	7	3	5	3	3	1
	21	2	2	3	3	2	5	1	1	3	4	3	3	1
	22	2	2	3	3	2	5	1	1	3	5	3	3	1
	23	2	2	3	3	2	5	1	2	3	5	2	2	1
	24	2	2	3	3	2	5	1	2	3	5	3	3	1
	25	2	2	3	3	2	5	1	4	3	4	3	3	1
	26	2	2	3	3	2	5	1	4	3	5	2	3	1
	27	2	2	3	3	2	5	1	5	3	4	3	3	1
	28	2	2	3	3	2	5	1	5	3	4	3	4	1
	29	2	2	3	3	2	5	1	5	3	5	2	3	1
	30	2	2	3	3	2	5	1	5	3	5	3	3	3
	31	2	2	3	3	2	5	1	6	2	5	3	3	1
	32	2	2	3	3	2	5	1	6	3	2	1	3	1
	33	2	2	3	3	2	5	1	6	3	5	3	2	2
	34	2	2	3	3	2	5	1	6	3	5	3	3	14
	35	2	2	3	3	2	5	1	6	3	5	3	4	1
	36	2	2	3	3	2	5	1	7	1	5	3	1	3
	37	2	2	3	3	2	5	1	7	3	1	2	3	1
	38	2	2	3	3	2	5	1	7	3	2	1	3	1
	39	2	2	3	3	2	5	1	7	3	2	2	1	1
	40	2	2	3	3	2	5	1	7	3	3	3	1	1

Table 2.3. *continued*

Families	Pattern No.	MIRU-VNTR loci												No.of isolates
		2	4	10	16	20	23	24	26	27	31	39	40	
Beijing														
	41	2	2	3	3	2	5	1	7	3	4	3	3	6
	42	2	2	3	3	2	5	1	7	3	5	1	3	4
	43	2	2	3	3	2	5	1	7	3	5	2	3	8
	44	2	2	3	3	2	5	1	7	3	5	3	1	1
	45	2	2	3	3	2	5	1	7	3	5	3	2	7
	46	2	2	3	3	2	5	1	7	3	5	3	3	58
	47	2	2	3	3	2	5	1	7	3	5	3	4	2
	48	2	2	3	3	2	5	1	7	3	5	4	3	3
	49	2	2	3	3	2	5	1	7	3	5	5	3	1
	50	2	2	3	3	2	5	1	7	3	6	3	1	1
	51	2	2	3	3	2	5	1	7	3	6	3	3	2
	52	2	2	3	3	2	5	1	8	3	5	3	1	1
	53	2	2	3	3	2	5	1	8	3	5	3	3	5
	54	2	2	3	3	2	6	1	1	2	4	2	4	1
	55	2	2	3	3	2	6	1	7	3	5	3	3	2
	56	2	3	3	3	2	5	1	7	3	5	3	2	1
CAS														
	57	1	2	4	4	2	5	1	8	3	5	3	4	1
	58	2	2	7	4	2	5	1	1	3	4	3	4	1
EAI														
	59	2	1	5	3	2	6	2	2	3	4	3	4	1
	60	2	2	4	1	2	5	1	2	3	5	3	3	1
	61	2	2	4	2	2	6	2	2	3	4	3	2	1
	62	2	2	4	3	2	4	2	2	3	4	2	2	1
	63	2	2	4	3	2	6	2	2	2	4	3	2	1
	64	2	3	4	3	2	4	2	2	3	5	2	3	1
	65	2	3	4	3	2	6	2	2	3	3	3	2	1
	66	2	3	4	3	2	6	2	2	3	4	3	4	1
	67	2	4	4	3	2	5	1	2	2	2	2	2	1
	68	2	4	4	3	2	6	2	2	1	5	3	2	1
	69	2	4	4	3	2	6	2	2	3	4	3	1	1
	70	2	5	3	3	2	4	2	2	3	6	3	3	1
	71	2	5	4	1	2	6	2	2	3	4	2	2	1
	72	2	5	4	1	2	6	2	2	3	5	3	4	1
	73	2	5	4	2	2	5	2	2	3	5	3	3	1
	74	2	5	4	2	2	5	3	2	3	2	3	3	1
	75	2	5	4	2	2	6	2	2	3	4	2	5	1
	76	2	5	4	3	1	6	2	2	3	4	3	2	1
	77	2	5	4	3	2	1	2	2	3	4	1	4	1
	78	2	5	4	3	2	1	2	2	3	5	1	3	1

Table 2.3. *continued*

Families	Pattern No.	MIRU-VNTR loci												No.of isolates
		2	4	10	16	20	23	24	26	27	31	39	40	
EAI														
	79	2	5	4	3	2	2	2	2	3	4	1	2	1
	80	2	5	4	3	2	3	2	2	3	5	3	3	1
	81	2	5	4	3	2	4	2	2	3	4	2	2	1
	82	2	5	4	3	2	4	2	2	3	5	2	2	1
	83	2	5	4	3	2	6	2	2	1	1	3	4	1
	84	2	5	4	3	2	6	2	2	1	4	2	2	1
	85	2	5	4	3	2	6	2	2	2	3	4	2	1
	86	2	5	4	3	2	6	2	2	3	2	3	2	1
	87	2	5	4	3	2	6	2	2	3	2	3	4	1
	88	2	5	4	3	2	6	2	2	3	4	1	2	1
	89	2	5	4	3	2	6	2	2	3	4	2	2	4
	90	2	5	4	3	2	6	2	2	3	4	3	1	2
	91	2	5	4	3	2	6	2	2	3	4	3	2	13
	92	2	5	4	3	2	6	2	2	3	4	3	4	2
	93	2	5	4	3	2	6	2	2	3	4	4	4	1
	94	2	5	4	3	2	6	2	2	3	5	1	4	1
	95	2	5	4	3	2	6	2	2	3	5	3	2	1
	96	2	5	4	3	2	6	2	2	3	5	4	4	1
	97	2	5	4	3	2	7	2	2	3	4	3	4	1
	98	2	5	5	3	2	6	2	5	3	4	3	2	1
	99	2	5	6	3	2	6	2	7	3	5	1	3	1
	100	2	6	4	1	2	5	1	2	3	5	3	3	1
	101	2	6	4	2	2	5	2	2	3	5	3	2	1
	102	2	6	4	2	2	5	2	2	3	5	2	3	2
	103	2	6	4	3	1	6	2	2	3	4	3	2	1
	104	2	6	4	3	2	6	2	2	3	4	3	2	3
	105	2	6	4	3	2	6	2	2	3	4	4	2	1
	106	2	6	4	3	2	6	2	2	4	6	3	5	1
	107	2	7	4	2	2	4	2	2	3	4	3	4	1
	108	2	7	4	2	2	5	2	2	3	5	3	3	1
	109	2	7	4	3	2	6	2	2	2	1	4	4	1
	110	2	7	4	3	2	6	2	2	3	4	3	4	1
	111	2	9	4	3	2	6	2	2	3	5	3	3	1
	112	3	2	4	2	2	5	2	2	3	5	3	3	1
	113	3	9	4	2	2	5	2	2	3	4	3	3	1
	114	2	5	4	3	1	12	2	2	3	4	2	3	1
	115	2	5	4	3	2	10	2	2	3	3	3	3	1
	116	2	1	4	3	2	4	2	2	3	5	3	3	1
	117	2	5	4	3	2	6	2	2	3	4	2	2	1

Table 2.3. *continued*

Families	Pattern No.	MIRU-VNTR loci												No.of isolates
		2	4	10	16	20	23	24	26	27	31	39	40	
Haarlem														
	118	2	2	0	3	2	5	1	4	3	3	2	3	1
	119	2	2	2	2	2	5	1	5	3	3	2	2	1
	120	2	2	2	3	2	4	1	5	3	3	2	3	1
	121	2	2	2	3	2	5	1	4	3	2	2	3	1
	122	2	2	2	3	2	5	1	5	1	3	2	3	1
	123	2	2	2	3	2	5	1	5	3	2	2	3	2
	124	2	2	2	3	2	5	1	5	3	3	2	3	6
	125	2	2	2	3	2	5	1	6	3	3	2	3	1
	126	2	2	2	4	2	5	1	4	3	3	2	4	1
	127	2	2	4	3	2	5	1	4	3	3	2	3	1
	128	2	2	5	1	2	5	1	1	3	3	2	2	3
	129	2	2	5	3	2	5	1	4	3	3	2	3	1
	130	2	2	5	3	2	5	1	5	3	3	2	3	2
	131	2	2	6	3	2	5	1	5	3	3	2	3	2
	132	2	2	6	4	2	3	1	5	3	3	2	4	1
	133	2	2	7	2	2	5	1	1	3	3	2	2	1
	134	2	3	2	3	2	5	1	6	3	3	2	1	1
	135	2	3	2	3	2	5	1	5	3	3	2	3	2
LAM														
	136	1	2	4	3	2	6	1	5	2	2	2	6	1
	137	2	2	2	2	2	4	1	5	3	2	2	3	1
	138	2	2	3	3	1	6	1	5	3	2	2	6	1
	139	2	2	4	4	2	4	1	4	3	2	2	1	1
	140	2	3	2	3	2	5	1	3	2	3	2	2	1
	141	2	4	2	3	2	5	1	5	2	2	2	1	1
S														
	142	2	2	3	2	2	5	1	7	3	4	3	3	1
	143	2	2	5	2	2	5	1	7	3	4	3	3	3
	144	2	2	6	2	2	5	1	7	3	4	3	3	1
	145	2	2	7	2	2	5	1	7	3	4	3	3	3
	146	2	2	7	2	2	5	1	7	3	4	3	4	1
	147	2	2	8	2	2	5	1	6	3	4	3	3	1
	148	2	2	8	2	2	5	1	7	3	4	2	2	1
	149	2	2	8	2	2	5	1	7	3	4	3	2	1
	150	2	2	8	2	2	5	1	7	3	4	3	3	1
	151	2	2	8	2	2	5	1	7	3	4	4	3	2
	152	2	2	9	2	2	5	1	7	3	4	3	3	1

Table 2.3. *continued*

Families	Pattern No.	MIRU-VNTR loci												No.of isolates
		2	4	10	16	20	23	24	26	27	31	39	40	
T														
	153	2	1	2	2	2	5	1	1	2	3	2	3	1
	154	2	2	2	2	2	5	1	1	3	3	2	2	2
	155	2	2	2	3	2	5	1	5	3	3	2	2	1
	156	2	2	2	4	2	5	1	6	3	3	2	2	1
	157	2	2	2	4	2	6	1	5	3	3	2	2	1
	158	2	2	3	3	2	5	1	5	3	3	2	1	1
	159	2	2	4	3	2	5	1	5	3	3	2	3	1
	160	2	2	5	1	2	5	1	1	1	2	2	2	2
	161	2	2	5	1	2	5	1	1	3	1	2	2	1
	162	2	3	4	3	2	5	1	5	2	3	3	2	1
	163	2	3	4	3	2	5	1	5	3	3	2	3	1
	164	2	4	2	3	2	4	1	1	2	3	2	2	1
	165	2	4	2	3	2	4	1	5	2	3	2	2	3
	166	2	4	2	3	2	4	1	5	3	3	2	1	1
	167	2	4	2	3	2	4	1	5	3	3	2	2	1
	168	2	4	2	3	2	5	1	2	2	3	2	2	2
	169	2	4	2	3	2	5	1	3	2	2	2	2	1
	170	2	4	2	3	2	5	1	4	2	3	2	3	1
	171	2	4	2	3	2	5	1	4	2	4	2	2	1
	172	2	4	2	3	2	5	1	5	2	3	2	1	1
	173	2	4	2	3	2	5	1	5	2	3	2	2	7
X														
	174	2	2	4	3	2	5	1	5	3	3	3	2	1
	175	2	2	4	3	2	5	1	6	3	3	2	4	1
	176	2	2	4	4	2	5	1	2	3	3	2	3	1

Appendix 3. Papers and manuscripts generated from this PhD thesis

1. **Sun YJ**, Bellamy R, Lee ASG, Ng ST, Ravindran S, Wong SY, Locht C, Supply P, Paton N. Use of mycobacterial interspersed repetitive unit-variable-number tandem repeat typing to examine genetic diversity of *Mycobacterium tuberculosis* in Singapore. Journal of Clinical Microbiology 2004, 42(5):1986-93.

2. **Sun YJ**, Lee ASG, Ng ST, Ravindran S, Kremer K, Bellamy R, Wong SY, van Soolingen D, Supply P, Paton N. Characterization of ancestral *Mycobacterium tuberculosis* by multiple genetic markers and proposal of genotyping strategy. Journal of Clinical Microbiology 2004, 42(11):5058-64.

3. **Sun YJ**, Lee ASG, Wong SY, Paton NI. Association of *Mycobacterium tuberculosis* Beijing genotype with tuberculosis relapse in Singapore. Epidemiology and Infection, 2006, 134(2):329-32.

4. **Sun YJ**, Lim TK, Ong A, Ho B, Seah GT, Paton NI. Tuberculosis associated with *Mycobacterium tuberculosis* Beijing and non-Beijing genotypes: a clinical and immunological comparison. BMC Infectious Diseases 2006, 6(1):105.

5. **Sun YJ**, Lee ASG, Wong SY, Heersma H, Kremer K, van Soolingen D, Paton NI. Genotype and phenotype relationships and transmission analysis of drug-resistant tuberculosis in Singapore. International Journal of Tuberculosis and Lung Diseases. Submitted.

6. **Sun YJ**, Lee ASG, Wong SY, Paton NI. Molecular and phylogenetic analysis of a novel family of *Mycobacterium tuberculosis* in Singapore. In preparation.

7. **Sun YJ**, Ong A, Lim TK, Ho B, Seah GT, Paton NI. Decreased interferon-γ and increased interleukin-4 expression in tuberculosis with pulmonary cavity. In preparation.